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EFFECT OF DIET ON EGG COMPOSITION

II. MORTALITY OF EMBRYOS IN EGGS FROM HENS ON DIETS CONTAINING PROTEIN SUPPLEMENTS OF DIFFERENT ORIGIN

By

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INTRODUCTION

BYERLY, Titus, and Ellis (19) found that eggs from hens fed different diets varied in hatchability and in the time of mortality of the contained embryos. Of the diets used, the ones containing only feedstuffs of vegetable origin yielded eggs in which a relatively large percentage of the embryos died during the second week of incubation. This high second week mortality was associated with a high incidence of chondrodystrophy, a type of defective cartilage and bone development described in chick embryos by Dunn (5) and Landauer (10). The experiments in the present paper extend and confirm the earlier report on the effect of diet on the ability of eggs produced to sustain embryonic life through the second week of incubation.

MATERIALS AND METHODS

The data to be presented were collected during the period between September, 1929, and August, 1932, inclusive. Data were gathered on time of embryonic mortality in the eggs from the breeding flocks at Beltsville, Maryland, during the breeding seasons of 1930, 1931, and 1932. The breeding flocks were housed in lots of 10 to 25 females and a single male in colony laying houses situated on plots of ground from about 900 sq. m. to about 2,000 sq. m. in area. The plots contained a variable amount of blue-grass

TABLE

EMBRYONIC MORTALITY, HATCHABILITY, AND CHONDRODYSTROPHY IN EGGS FROM PRESENTED FOR

Diet	Birds	Fertile eggs set	First week		Second week	
			Mortality	Difference normal and other diets	Mortality	Difference normal and other diets
	No.	No.	Per cent	Per cent	Per cent	Per cent
Basal	130	2563	10.3±0.70	+0.7±0.92	13.5±1.16	+10.9±1.21
Cottonseed meal	46	698	12.6±1.14	+3.0±1.28	17.2±1.96	+14.6±1.99
Soybean meal	77	2359	13.4±0.91	+3.8±1.13	18.7±1.65	+16.1±1.68
Ground soy beans	38	1587	9.3±0.91	−0.3±1.13	11.0±1.47	+8.4±1.50
Yeast preparation	32	500	5.8±0.81	−3.8±1.01	6.0±1.09	+3.4±1.14
Meat meal	65	1241	15.2±1.31	+5.6±1.44	10.7±1.20	+8.1±1.24
Menhaden fish meal	13	262	6.7±1.65	−2.9±1.75	13.3±3.06	+10.7±3.08
North Atlantic fish meal	47	840	14.4±1.40	+4.8±1.52	3.2±0.78	+0.6±0.84
Crabscrap meal	47	962	9.2±0.81	−0.4±1.00	2.8±0.45	+0.2±0.55
Buttermilk	30	601	8.6±0.75	−1.0±1.06	3.6±0.63	+1.0±0.71
Normal	111	4898	9.6±0.59	—	2.6±0.32	—
Breeding flocks Reds and Leghorns only	423	8752	10.1±0.39	—	2.3±0.15	—
Breeding flocks total	1156	23880	11.4±0.25	—	2.6±0.11	—

TABLE

EMBRYONIC MORTALITY, HATCHABILITY, AND CHONDRODYSTROPHY IN EGGS FROM YEARLING IN CONFINEMENT AND

Diet	Birds	Fertile eggs set	First week		Second week	
			Mortality	Difference pullet year diet and others	Mortality	Difference pullet year diet and others
	No.	No.	Per cent	Per cent	Per cent	Per cent
Pullet year: various diets	90	1850	22.3±1.56	—	30.5±1.36	—
Soy bean-cotton-seed	10	158	20.7±5.10	−1.6±5.33	37.6±7.62	+7.1±7.74
Basal	42	2513	17.1±1.89	−5.2±2.45	17.4±1.72	−13.1±2.19
Normal	25	1007	11.5±1.32	−10.8±2.56	4.3±0.82	−26.2±1.59

I

PULLETS ON VARIOUS DIETS WHEN FED IN CONFINEMENT; BREEDING FLOCK DATA COMPARISON

Third week		Chicks hatched		Chondrodystrophy	
Mortality	Difference normal and other diets	Total	Difference normal and other diets	Incidence	Difference normal and others diets
Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
17.2±0.84	−0.9±1.14	59.0±1.62	−10.7±1.86	7.45±0.84	+7.23±0.85
15.1±1.50	−3.0±1.68	55.1±2.81	−14.6±2.95	6.06±1.13	+5.84±1.13
18.5±0.98	+0.4±1.25	49.4±1.90	−20.3±2.21	5.05±0.56	+4.83±0.57
20.2±1.43	+2.1±1.62	59.5±2.59	−10.2±2.84	1.61±0.39	+1.39±0.40
18.0±1.54	−0.1±1.72	70.0±2.38	+0.3±2.74	1.60±0.52	+1.38±0.53
16.6±0.92	+1.5±1.20	57.5±2.21	−12.2±2.39	2.82±0.50	+2.60±0.51
30.7±3.72	+12.6±3.80	49.3±4.19	−20.4±4.29	2.00±0.78	+1.78±0.79
16.3±1.07	−1.8±1.33	66.1±1.87	−3.6±2.08	0.30±0.12	+0.08±0.14
15.9±1.14	−2.2±1.38	72.1±1.90	+2.4±2.21	0.13±0.087	−0.09±0.12
13.1±1.62	−5.0±1.79	74.7±1.86	+5.0±2.07	0.17±0.11	−0.05±0.13
18.1±0.77	—	69.7±0.91	—	0.22±0.074	—
13.3±0.39	—	74.3±0.60	—	0.017±0.105	—
17.6±0.30	—	68.4±0.42	—	0.28±0.039	—

II

HENS WHICH PRODUCED CHONDRODYSTROPHIC EMBRYOS DURING THEIR PULLET YEAR, KEPT FED VARIOUS DIETS

Third week		Chicks hatched		Chondrodystrophy	
Mortality	Difference pullet year diet and others	Total	Difference pullet year diet and others	Incidence	Difference pullet year diet and others
Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
15.2±0.80	—	32.0±1.12	—	16.9 ±1.05	—
18.0±3.27	+2.8±3.36	24.0±7.82	−8.0±7.90	20.2 ±5.90	+3.3±5.99
13.8±1.00	−1.4±1.28	51.7±2.50	+19.7±2.74	1.68±0.37	−15.22±1.11
26.9±2.52	−11.7±2.64	57.3±2.86	+25.3±3.07	0.88±0.72	−16.02±1.13

TABLE

EMBRYONIC MORTALITY, HATCHABILITY, AND CHONDRODYSTROPHY IN EGGS FROM

Diet	Birds	Fertile eggs set	First week		Second week	
			Mortality	Difference confined birds on basal diet and others	Mortality	Difference confined birds on basal diet and others
	No.	No.	Per cent	Per cent	Per cent	Per cent
Basal	41	875	6.5±0.80	−3.8±1.06	0.6±0.20	−12.9±1.18
Soybean meal	42	832	7.1±0.91	−3.2±1.15	2.8±0.87	−10.7±1.45
Linseed meal	41	767	6.4±0.81	−3.9±1.07	1.3±0.35	−12.2±1.21
Peanut meal	38	696	4.3±0.74	−6.0±1.01	2.4±0.79	−11.1±1.41
Breeding flock Giant-Leghorn F_1 only	109	1465	7.6±0.54	—	1.2±0.26	—

sod and were partially shaded. The breeding of the birds and evidence of genetic causes for differences within the breeding flock in time of embryonic mortality have been discussed by Byerly, Knox and Jull (3). The diet given the breeding flocks contained whole corn, wheat, and a small amount of semi-solid buttermilk in addition to the constituents of the normal diet fed confined birds. Eggs were incubated from about February 15 to May 1. These data are introduced to furnish a normal mortality curve for basic comparisons and to demonstrate the adequacy of the normal diet used for the confined birds to enable the production of eggs capable of sustaining embryonic life through the second week of incubation in normal fashion.

Portions of these breeding flock data have been statistically treated and included in Tables I and III for the same purpose.

The conditions of housing, management, and selection of experimental material for the experiments with confined birds on the "normal" diet and the composition of the diet have been described in some detail by Byerly, Titus, and Ellis (2).

The data on confined birds contained in this paper were obtained from experiments begun in September, 1929, 1930, and 1931. Each experiment covered a period of eleven months and some or all of the eggs produced each month were incubated. Pens of Single Comb Rhode Island Red and Single Comb White Leghorn pullets were used except in the experiment described in Table II for which Single Comb Rhode Island Red yearlings which produced chondrodystrophic embryos during their pullet year on experimental diet were used.

III

PULLETS FED ALL-PLANT SOURCE DIET, WITH ACCESS TO BLUE-GRASS RANGES

Third week		Chicks hatched		Chondrodystrophy	
Mortality	Difference confined birds on basal diet and others	Total	Difference confined birds on basal diet and others	Incidence	Difference confined birds on basal diet and others
Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
15.0±1.16	−2.2±1.43	77.9±1.27	+18.9±2.05	0.0	−7.23
19.6±1.49	+2.4±1.71	70.5±2.03	+11.5±2.60	0.14±0.098	−7.09±0.85
23.2±1.53	+6.0±1.74	69.1±1.57	+10.1±2.25	0.15±0.10	−7.10±0.86
21.3±1.69	+4.1±1.89	72.0±1.73	+13.0±2.37	0.0	−7.23
26.4±1.15	—	64.8±1.33	—	0.0	—

Byerly, Knox and Jull (3) have described modifications in the curve for embryonic mortality due to genetic differences in the Beltsville flock, and since they found no significant difference between the second week mortality of embryos from Leghorns and Rhode Island Reds, and since no significant differences attributable to breed exist in the experimental data concerning second week mortality in these experiments, data from Rhode Island Red and Leghorn pens have been summarized together in the present paper.

The diets fed the confined birds used in the experiments summarized in Table I were compounded as follows:

Basal feed mixture:	Parts, by weight
Yellow corn meal (ground yellow corn)	50
Wheat bran	24.5
Oat meal	15
Alfalfa leaf meal	5.5
Mineral mixture (see text)	
Cod-liver oil	2
Protein supplement	20

The mineral mixture consisted of such amounts of ground limestone and steamed bone meal as were required to give the desired Ca/P ratio which was from 2:1 to 4:1 in the various experiments; 0.5 part of common salt and 0.5 part of anhydrous sodium sulphate. The basal diet of course was as described except that it contained 20 parts of the basal feed mixture instead of the protein supplement.

Other lots of birds were used to test the value of bluegrass range as a supplement to all-vegetable diets; to test various feedstuffs for toxicity; to test the effects of various Ca/P ratios on second week mortality and incidence of chondrodystrophy and to test the deficiency of individual feedstuffs when fed alone. Material and methods used in these experiments are described later in this paper.

Mortality Curves

Four typical mortality curves are given in Figure 1 to demonstrate the sharply defined peaks in mortality more clearly than can be done in the tables. Diets described as giving high second-week mortality of embryos all gave the sharp peak, or a more pronounced one, shown for the birds on the basal diet fed in confinement in Figure 1A.

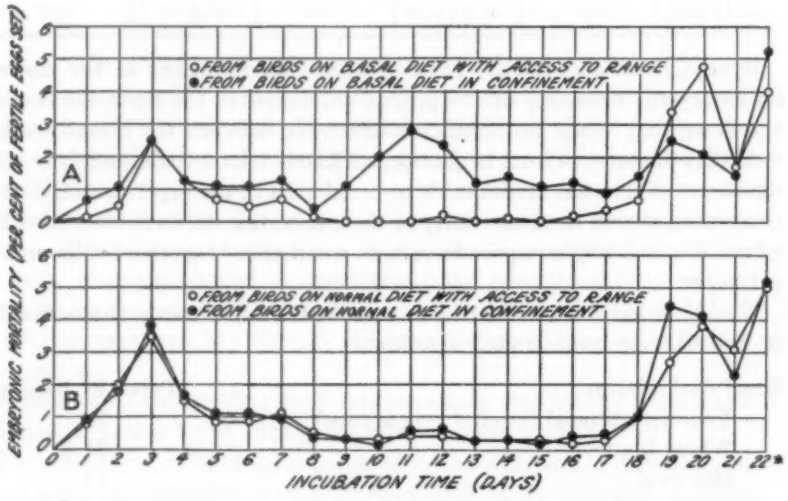
EMBRYONIC MORTALITY (PER CENT OF FERTILE EGGS SET)
o FROM BIRDS ON BASAL DIET WITH ACCESS TO RANGE
● FROM BIRDS ON BASAL DIET IN CONFINEMENT
A
o FROM BIRDS ON NORMAL DIET WITH ACCESS TO RANGE
● FROM BIRDS ON NORMAL DIET IN CONFINEMENT
B
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22*
INCUBATION TIME (DAYS)

FIG. 1.—Embryonic mortality curve as affected by variations in diet of the parent stock.

A.—o. Mortality of embryos in eggs from birds receiving the basal diet with access to bluegrass range.

● Mortality of embryos in eggs from birds receiving the basal diet in confinement.

B.—o. Mortality of embryos in eggs from the breeding flocks.

● Mortality of embryos in eggs from birds receiving the normal diet in confinement.

* Alive on the 22nd day of incubation but not hatched.

The percentage mortality of fertile eggs on each day of the incubation period is plotted against incubation time in Figure 1B for the breeding flock data. The mortality curve so obtained is very similar to that pub-

lished by Payne (16) for the fowl and by Riddle (18) for the dove. It does not contain a peak during the second week of incubation which Byerly (1) reported as of frequent occurrence. Byerly based his statement at that time on data from both confined and free range birds though the significance of this fact was not then appreciated.

The curve for embryonic mortality in eggs produced by hens receiving a "normal" diet in confinement during the three years of experiment is plotted in Figure 1B as well. There is very close agreement between this curve and the curve for embryonic mortality in embryos from the breeding flocks. This demonstrates the adequacy of the normal diet used for the production of eggs capable of sustaining embryonic life in normal fashion.

The mortality curve for embryos in eggs from confined birds on the basal diet is given in Figure 1A. It will be noted that a relatively large portion of the mortality occurred during the second week of incubation, during which period the mortality was very low in embryos in eggs from the breeding flocks and confined birds on the normal diet. A mortality curve for embryos in eggs from birds on a similar basal diet, to be described later, but with access to range is also plotted in Figure 1A. This curve lacks the high second-week mortality shown in the curve for embryos in eggs from birds fed the basal diet in confinement. This demonstrates that some feed or feeds obtained from the range used are capable of supplying a deficiency existing in the basal diet. The protein concentrate in the normal diet, consisting of 8 parts meat meal, 7 parts North Atlantic fish meal, and 5 parts of dried buttermilk, is shown to satisfy this deficiency by the mortality curve in Figure 1B.

Effects of Protein Concentrates on Embryonic Mortality and Chondrodystrophy

Table I contains statistical comparisons[1] between the relative embryonic mortality in eggs from hens on several diets expressed as per cent of fertile eggs set. That portion of the data from the breeding flocks which was derived from the Single Comb Rhode Island Red and Single Comb Leghorn flocks of the same breeding as the experimental birds is included for comparison.

The difference in mortality, in hatchability of fertile eggs set, and in percentage of chondrodystrophy among fertile eggs set, in each period, between the normal and each of the other diets, are given with their probable

[1] The probable error of the mean of the values for the hens on each of the diets and the probable error of the differences between those means are the statistical measures of significance used.

TABLE

EMBRYONIC MORTALITY, HATCHABILITY, AND CHONDRODYSTROPHY

	Diet	Birds	Eggs	First week		Second week	
				Mortality	Difference corn and other diets	Mortality	Difference corn and other diets
		No.	No.	Per cent	Per cent	Per cent	Per cent
Corn	Reared and fed	8	227	23.3±3.56	—	11.6±2.46	—
	Fed	5	332	39.8±10.01	—	13.6±1.31	—
Wheat	Reared and fed	9	534	25.8±2.37	+2.5±4.27	44.2±5.62	+32.6±6.13
	Fed	4	115	96.8±1.48	+57.0±10.1	1.3 ±0.63	−12.3±1.45
Soy bean	Reared and fed	1	18	16.7	−6.6	16.7	+5.1
	Fed	3	90	64.6±5.94	+24.8±11.7	12.7±0.20	−0.9 ±1.33
Buttermilk	Fed	2	32	77.5±0.34	37.7±10.0	0±0.0	−13.6

error. These values indicate the probable effect of diet on each of the factors named. The same method of comparison is used for Tables II, III, and IV with other appropriate diets as the base of comparison in each case.

The mortality during the first week of incubation was significantly greater than that on the normal diet in eggs from birds receiving the basal diet supplemented with soybean meal, with meat meal, and with North Atlantic fish meal; significantly lower first-week mortality was obtained only on the diet containing the yeast preparation. These differences are all relatively small, 58 per cent or less of the value for eggs from hens on the normal diet, whereas differences in second-week mortality are relatively great being about 620 per cent of the normal in case of the eggs from birds receiving soybean meal.

It is evident that the mixture of protein concentrates used in the normal diet, the buttermilk, crab meal, and North Atlantic fish meal have satisfied the deficiency in the basal diet. Eggs from birds receiving diets containing these supplements supported life during the second week of embryonic development as well as did the eggs from birds in the breeding pens which

IV

IN EGGS FROM PULLETS RECEIVING A SINGLE SOURCE OF PROTEIN

Third week		'Chicks hatched		Chondrodystrophy	
Mortality	Difference corn and other diets	Total	Difference corn and other diets	Incidence	Difference corn and other diets
Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
16.0±3.27	—	49.1±3.96	—	2.88±0.87	—
24.8±4.32	—	21.8±5.87	—	0.80±0.95	—
13.8±2.47	−2.2±4.09	16.2±2.47	−32.9±4.66	10.3±1.67	+7.42±1.89
1.2±1.06	−23.6±4.45	0.7±0.63	−21.1±5.90	0.0	−0.80
5.6	−10.4	61.0	+11.9	0.0	−2.88
18.7±3.64	+2.7±4.40	4.0±2.00	−17.8±6.20	0.0	−0.80
12.5±1.01	−3.5±2.48	10.0±0.67	−11.8±5.91	0.0	−0.80

were kept on range and fed a normal diet. The other diets all gave significantly higher second-week mortality and incidence of chondrodystrophy than the normal diet except the diets containing Menhaden fish meal and the yeast preparation. The Menhaden diet was fed to only a small lot of birds; embryonic mortality in the eggs from these birds was significantly higher than normal during the second week of incubation while the incidence of chondrodystrophy was higher but not significantly so, possibly owing to the small number of birds used. The yeast preparation may have had some supplemental value; more data are needed.

The results obtained with meat meal were quite variable. During the first year the diet gave fairly good results, as reported by Byerly, Titus, and Ellis (2) though the incidence of chondrodystrophy was higher than that in eggs from birds receiving other animal protein concentrates during the same year. During the 1930–31 experiment, the pens receiving meat meal gave poor results. The difference, in the authors' opinion, is probably due to differences in treatment of the meat meal during the process of manufacture.

It will be noted that ground whole soy beans gave a lower second-week mortality than soybean meal, the difference being 7.7 ± 2.22 per cent, although second-week mortality in eggs from the ground soy bean lot was significantly higher than that in eggs from birds on the normal diet. One lot of birds receiving ground soy beans of a small black variety (Wilson) gave results equal to those obtained from the normal diet. The soybean meal and the other lots of ground soy beans were manufactured from a larger, yellow variety of bean (Mammoth Yellow).

Third week mortality differed significantly from the normal only in the case of eggs from the birds on Menhaden fish meal. This striking uniformity in third-week mortality is only apparent because, of course, in those eggs from groups showing high mortality during the first two weeks, there were relatively fewer live embryos during the third week. The uniformity in percentage mortality during the third week of incubation therefore means that mortality during the third week of incubation was proportional to mortality during the first two weeks. This relatively increased mortality was probably indirectly due to the same cause or causes as the second-week mortality. Second-week mortality seems thus far to be more or less specifically related to the diet of the hen while mortality during the first and third weeks of incubation varies with many environmental factors other than diet.

It has been pointed out by Byerly, Titus, and Ellis (2) that high second-week mortality and chondrodystrophy are associated. It now seems likely from the work of Landauer (11) on the development of an inherited type of chondrodystrophy (the Creeper variety of fowl) that chondrodystrophy is a direct result of retardation of the growth of the embryo early in incubation. It is likely that the high incidence of chondrodystrophy in some of the present experiments only indicates that the deficiency in the eggs was operative at the critical period of growth described by Landauer. Whatever the nature of the deficiency that caused high second-week mortality, the same deficiency probably caused the associated high incidence of chondrodystrophy in these experiments.

Byerly, Titus, and Ellis (2) confirmed the Hutt and Greenwood (8) report that the incidence of chondrodystrophy cannot be attributed to chance. They also showed that the incidence of chondrodystrophy was apparently greater in the progeny of hens receiving feedstuffs of vegetable origin than in those containing an animal protein concentrate. It is apparent from the data in Table I that the birds on diets of vegetable feedstuffs have shown a relatively high incidence of chondrodystrophy

throughout the three years of the experiment. The meat meal diet gave a higher incidence during the subsequent experiments than in the first year although it was at that time noted that the meat meal diet gave the highest incidence of chondrodystrophy of any of the animal protein-containing diets used in that year. Menhaden fish meal seems to fall in the same class with meat meal. It was used only during the second year of the experiment.

Hen Variation and Chondrodystrophy and Embryo Mortality

In order to test further the effect of diet on incidence of embryo mortality during the second week of incubation and on the incidence of chondrodystrophy, pullets which produced such embryos during the experimental periods of 1929–30 and 1930–31 were segregated in the following experimental period. If there is any inherent tendency of certain birds to produce such embryos, these birds should give more convincing evidence of the role of diet than an unselected group.

The data obtained from these birds are summarized in Table II. It will be noted that the second-week mortality of embryos from hens on the normal diet is not significantly different from that of embryos from unselected pullets on the same diet (Table I), the difference being 1.7 ± 0.93. The embryos from birds on the basal diet showed a significantly higher second-week mortality than those on the normal diet, the difference being 13.1 ± 1.91. The incidence of chondrodystrophy was significantly decreased on both the basal and the normal diet from that of the progenies of the same birds in the preceding year. The incidence of chondrodystrophy and of second-week mortality on the basal diet are not significantly higher than those shown by unselected pullets on the same diet. This probably means that the evidence adduced by Hutt and Greenwood (8) and Byerly, Titus, and Ellis (2) that chondrodystrophy is significantly more frequent in the progenies of some birds than others indicates a temporary physiological idiosyncracy of those birds rather than a constant or inherent tendency toward the production of chondrodystrophic embryos.

The group indicated as cottonseed meal-soybean meal received cottonseed meal for six months of the period, then soybean meal for the remainder. These products were substituted for 40 parts of the basal feed mixture. This lot, though small, confirms the results obtained with these same products with pullets. There was no apparent difference between the data from the two portions of the experimental period so they were combined.

It has been noted that the chondrodystrophy-producing hens which re-

ceived the normal diet, produced eggs which sustained life through the first two weeks of incubation to a degree not significantly different from that of eggs produced by unselected pullets on the same diet. The third week mortality, however, is significantly greater in the eggs from the chondrodystrophy-producing hens. This may be due to the large size of the eggs laid by hens on this diet due to its high protein content of about 21 per cent, or to some other factor impossible to determine from the data at hand.

All Plant Source Diets and Range

Table III contains data from range birds. All birds fed the diets indicated were F_1 pullets from a cross between Jersey Black Giants and Single Comb White Leghorns. Experimental flocks received basal, soybean meal, linseed meal, and peanut meal diets, and in addition there were breeding flocks which received a normal diet. The birds were housed in colony laying houses in lots of 25 pullets. Each house was located in a plot of ground about 900 sq. m. in area covered with weedy blue grass. Cockerels of the same breeding as the pullets were used and were rotated from pen to pen each day among the experimental flocks. The basal diet used for one series of pens was that used for the confined birds. The basal for the duplicate series contained ground wheat and middlings instead of bran, and ground oats instead of oatmeal. Since no statistically significant differences were found between similar pens, they have been summarized together.

These eight pens of birds fed all-plant source diets gave good results. The data indicate that the birds were able to obtain from the range enough of the material lacking in the diet to keep the mortality of their embryos during the second week at a normal figure. The data from birds of the same breeding in the breeding pens show a higher third-week mortality. The latter birds were pen mated and the difference in third-week mortality is probably due to the consanguinity of the males and the females.

Non-toxicity of Feedstuffs Permitting High Second-week Mortality and Chondrodystrophy

In order further to test the properties of feedstuffs known to permit high second-week mortality of embryos, data were gathered from lots of confined birds fed the normal diet supplemented by 20 per cent of wheat bran, rice bran, and alfalfa-leaf meal, respectively. (Rice bran had been found to permit high second-week mortality of embryos when used with diets not discussed in the present paper.) None of these pens gave second-week mortality of embryos significantly different from that obtained in

embryos in eggs from birds on the normal diet. It may be concluded, therefore, that there is no toxic substance present in these feedstuffs that is responsible for this type of embryo mortality.

Variation in Mineral Portion of Diet Without Effect

Two pens of confined birds, not included in the data in Table I, were fed the normal diet except that the Ca/P ratio was adjusted to 1:1. Second week mortality was not significantly different from that in eggs from birds receiving the normal diet with a Ca/P ratio of 2:1 or 4:1. One pen of confined birds, not included in the data in Table I, received the basal diet with a supplement of 5 parts of dried kelp. The second-week mortality was not significantly different from that in eggs from birds receiving the unsupplemented basal diet.

Certain Protein Sources Permitting Growth and Egg Production are Inadequate for Reproduction

Data from experiments designed to give evidence as to the degree of the deficiency of some of the constituents of the several diets are given in Table IV. Chicks were reared on a sole diet of corn products, wheat middlings, and ground whole soy beans with dextrin filler, respectively, to find out whether these feedstuffs used alone would give the same or higher second-week mortality and incidence of chondrodystrophy as when used with other feedstuffs as described above. Only one pullet lived to lay eggs in the soy bean lot; so the data from that source are very meagre.

The birds used in this set of experiments were Single Comb White Leghorns. Those reared and fed on wheat middlings received only wheat middlings, cod-liver oil, and a mineral mixture containing calcite, bone meal, salt, and sodium sulphate. They had access to sunlight in a concrete floored yard but had no access either to green feed or the soil. The lot of birds reared and fed on corn, received a mixture of corn meal and corn-gluten meal blended to form a mixture with the same crude protein content as the wheat middlings. This pen received cod-liver oil and mineral mixture, and the breeding and housing were the same as for the middlings pen. Ground soy beans and dextrin, with mineral supplement and cod-liver oil, were used for the soy bean pen. The Ca/P ratio was adjusted to 1.8 to 1 in all three diets.

Corn and whole soy beans are apparently about as deficient as the basal diet, while wheat middlings gave much poorer results, apparently being more deficient than corn or soy beans. Four small supplemental pens of

cross-bred pullets (F_1 from Single Comb Rhode Island Red male × White Rock females) well-grown, sexually mature, and vigorous, were placed, one lot of 6 birds on each of the corn, wheat, and soy bean diets, and a fourth lot of 6 birds on a diet of buttermilk, dextrin, mineral mixture, and cod-liver oil. Again the corn diet was better than the wheat middlings diet from the standpoint of embryo mortality. Few of the embryos in eggs from the birds on the wheat middlings diet lived beyond the first three days of incubation. The data from the milk-dextrin diet are so scanty as to demonstrate only the possibility of hatching chicks from eggs produced on such a diet. It should be noted that the few eggs produced on this diet were laid after the birds had received the diet for six months or more.

Discussion

The data demonstrate clearly that the basal diet used is deficient in some substance or substances necessary for, or at least auxiliary to, embryonic life during the second week of incubation.

This deficiency can hardly be associated with fat or fat-soluble vitamin, for it was eliminated by feeding North Atlantic fish meal, buttermilk, and crab meal, all products of low fat content. Further, all diets used were supplemented with cod-liver oil and all birds had access to sunlight. All diets, except those containing but a single protein source, contained about 50 per cent yellow corn so that vitamin A should not be a limiting factor. Ellis, Miller, Titus, and Byerly (6) have shown adequate vitamin A content of eggs from hens receiving the same cod-liver oil and more deficient diets.

The deficiency cannot be ascribed to improper Ca/P ratio for this was varied from 1:1 to 4.0:1 without correlated changes in second-week mortality or incidence of chondrodystrophy. The same is true for amount of Ca and P for these were varied fairly widely without correlated changes in second-week mortality. The deficiency is probably not satisfied by iodine as present in kelp.

The water soluble vitamins B and G, as at present established, may not be completely ruled out but the evidence is against them. Ellis, Miller, Titus, and Byerly (6) have shown that the vitamin B content of eggs may be varied by diet but that chicks may hatch from vitamin B deficient eggs though they often die of polyneuritis soon afterward. No cases of polyneuritis in chicks hatched from eggs described in the present experiment were observed nor would the constitution of the diets themselves indicate the possibility of vitamin B deficiency.

The experiments of Ellis, Miller, Titus, and Byerly (6) do not indicate pronounced decrease in the vitamin G content of eggs produced on diets very deficient in this factor as compared to the normal diet. On the other hand, some supplements which overcome the deficiency in the basal diet are substances which are fairly rich in vitamin G. This is especially true of the range birds and of those receiving buttermilk. It is possible that vitamin G or some other vitamin plays an indirect role, permitting better assimilation of materials not otherwise utilized.

The qualitative protein content of the diet itself may be the effective factor. Evidence on the possibility of varying the quality of the protein content of eggs is thus far on a somewhat insecure basis. McFarlane, Fulmer, and Jukes (13) were unable to show variation in amino acid distribution due to diet. On the other hand, Pollard and Carr (17) and Gerber and Carr (7) have reported data on pigeon eggs which they interpreted as showing that amino acid distribution in such eggs may be affected by diet and that such changes affect the hatchability of pigeon eggs. Titus, Byerly, and Ellis (19) have reported data which they interpreted as indicating differences in crude protein content of eggs due to diet fed the hens producing them. Calvery and Titus (4) were unable to find differences in the corn and wheat diet eggs described in these experiments as to their amino acid makeup. Much work must be done both as to the physical chemistry and organic chemistry of egg proteins before a final conclusion may be drawn as to the effect of diet.

A considerable mass of data does exist, however, that indicates a possible explanation of the failure of some of the protein concentrates to satisfy the deficiency of the basal diet. The work of Maynard (12) on fish meals indicates clearly that the process of manufacture of Menhaden fish meal by the flame-drying process leads to considerable decrease in the biological value of the protein. Other conditions of manufacture of this product probably also affect it adversely. Ingvaldsen (9) has reported destruction of amino acids in Menhaden fish meal by heat.

This effect of heat probably applies to proteins generally. Morgan (15) showed that cereal proteins are affected even by the heat used in the toasting process.

The meat meals used were dry rendered and so also heated to an unknown and probably variable temperature. It is possible that the process of oil extraction from soy beans and cottonseed also caused deterioration of the proteins and this may account for the difference noted between the effectiveness of whole ground soy beans and soybean meal.

The argument may of course be advanced that the reported biological values of the proteins used do not support an hypothesis that qualitative protein deficiences are in any way responsible for the high second week mortality of embryos and its associated high incidence of chondrodystrophy. Tests of biological value of proteins are not necessarily applicable to experiments on reproduction. Diets adequate for growth and maintenance are not necessarily adequate for reproduction as pointed out by Mitchell (14). In the present experiments, the birds reared on wheat middlings grew faster and appeared to be in better physical condition than those reared on corn products; yet eggs from the latter were distinctly superior for the maintenance of embryonic life.

The period of incubation during which mortality was specifically increased itself suggests some derangement of protein metabolism, for it is in the first half of the incubation period that protein is metabolized in greatest amounts relative to the amount of living material in the egg. Further, many of the dead and moribund embryos were highly edematous.

Many deficiencies in diet and what has been referred to as deficiency in these experiments may be deficiency only in the sense that some of the experimental animals receiving such diets were unable properly to assimilate necessary materials actually present in the diet. This seems to have been true of the birds which produced chondrodystrophic embryos as previously noted. Further, eggs from birds reared on corn, middlings and soy beans, respectively, were less deficient than eggs from birds given these same feeds only after they were well grown. This must mean that among

TABLE V

VARIATION IN EGG PRODUCTION AND HATCHABILITY OF EGGS PRODUCED BY BIRDS IN CONFINEMENT RECEIVING THE NORMAL AND BASAL DIETS, RESPECTIVELY, FOR ONE ENTIRE ELEVEN-MONTH PERIOD

Diet	Birds	Hatchability	Coefficient of variation	Egg production	Coefficient of variation	Correlation coefficient egg production and hatchability
	No.	Per cent		No.		
Basal.....	164	56.8	48.1±1.99	104.7	32.5±1.33	+0.155 ±0.0768
Normal.....	151	66.4	27.2±1.13	148.1	25.8±1.06	+0.136 ±0.0803
Difference....	—	—	20.9±2.29	—	6.7±1.71	—

the birds *reared* on these feeds, those birds less able to utilize them died before reaching maturity.

In Table V data are tabulated from birds which survived an entire experimental period on the normal and on the basal diet, respectively. The coefficients of variability for the hatchability of eggs and for the number of eggs produced are higher for the birds on the basal diet. This is the expected result since increased variability in experimental animals is in itself often considered an evidence of inadequate diet.

Correlation between egg production and hatchability is positive but not significant for the birds on each diet. This means that the birds varied in the efficiency with which they transferred materials necessary for embryonic growth from the diet to the egg. The birds which laid few eggs were not able to store materials present in minimal quantities and thus to produce better eggs; if anything, those that were efficient, able to convert larger amounts of feed into eggs, even though the feed supplied was deficient, were also able to produce eggs more capable of supporting embryonic life. This is a very important matter from the practical standpoint for the opinion is popularly held that the barnyard fowl, which produces few eggs, must consequently produce better eggs from the standpoint of human nutrition than the bird bred and fed to lay many eggs. If the ability of an egg to sustain embryonic life is an index of its value as human food then the eggs from the efficiently managed high-producing flocks are at least as desirable as any others.

It is recognized, of course, that treatment in the process of manufacture, especially with heat, may reduce the vitamin potency of the protein concentrates used. In the case of the vitamin G potency of meat meal this must be true for, whereas lean meat is supposedly a fair source of vitamin G, the experiments of Ellis et al., show that the meat meal used in these experiments was very deficient.

Conclusions

Diets consisting of certain cereal products supplemented with alfalfa-leaf meal, minerals, and cod-liver oil caused the production of eggs the embryos in which showed a significantly greater mortality during the second week of incubation than the embryos in eggs from birds on a diet supplemented with certain protein concentrates of animal origin.

It is concluded that corn, wheat, ground soy beans, soybean meal, cottonseed meal, flame-dried Menhaden fish meal and some lots of meat meal are deficient in some substance necessary for the production of eggs

capable of supporting embryonic life during the second week of incubation.

It seems likely that factors in the process of manufacture of the deficient protein concentrates, especially excessive heat, may be responsible for their deficiency.

Vacuum dried North Atlantic fish meal, steam-dried crab meal, buttermilk, a combination of buttermilk, North Atlantic fish meal, and meat meal, and free range were found to enable the production of eggs capable of supporting embryonic life through the second week of incubation in normal fashion.

Evidence as to the nature of the deficiency is somewhat conflicting. The proteins in the deficient concentrates may well have been qualitatively deficient. It is possible that vitamin G or some one of the less well known water soluble vitamins is concerned although the authors have thus far been unable to obtain experimental evidence for such a view.

There was no tendency for the birds that laid fewer eggs when receiving deficient diets to lay eggs more capable of sustaining embryonic life than those laid by birds laying a greater number while receiving the same deficient diet.

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EFFECT OF DIET ON EGG COMPOSITION

III. THE RELATION OF DIET TO THE VITAMIN B AND THE VITAMIN G CONTENT OF EGGS, TOGETHER WITH OBSERVATIONS ON THE VITAMIN A CONTENT

By

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THIS paper presents a series of vitamin investigations which have formed a part of a study on the effect of diet on the production, size, composition, vitamin content, nutritive value, and hatchability of eggs. Papers on the chemical composition of the eggs and on the embryonic mortality have already appeared (1, 2, 3). The present studies have been directed at the influence of the diet on the content of the egg in the factors associated in the vitamin B complex. Particular attention has been given to the vitamin B and vitamin G content of eggs produced on diets which showed a considerable variation in these vitamin factors and in hatchability. Because of a wide difference in pigmentation of the eggs produced on the different diets, all of which contained cod-liver oil, the vitamin A content was determined on a normally pigmented lot of eggs in comparison with another lot nearly devoid of yellow pigments.

Recent investigations (4) have confirmed earlier reports that vitamin G (B_2) as well as vitamin B (B_1) is essential for the growth of the chick while the B_3 factor apparently is not essential.

While the vitamin content of eggs, like that of milk, possibly is not affected by external factors to the extent that plant materials may be affected (5), alterations in the supply of vitamin A and of vitamin D in the diet of the hen have been found to influence the composition of the egg (6). Likewise direct irradiation of hens may improve the hatchability of the eggs (7, 8).

In the usual case the work on the vitamin B complex and on vitamins B and G has been directed toward the relative richness of eggs in the sev-

eral factors rather than toward the effect of the diet of the hen on the eggs. Steenbock (9) prepared a water-acetone-soluble fraction from egg yolk, small doses of which cured pigeons suffering from polyneuritis. Osborne and Mendel (10) in feeding tests with rats showed that eggs were approximately three times as potent in vitamin B (complex) as milk. Hoagland and Lee (11) used pigeons to determine the amount of the antineuritic factor in eggs. They found that a diet of polished rice and dried eggs did not fully protect against polyneuritis even when the eggs were fed at a 30 per cent level. They cited the work of Cooper (12) as confirming their results which indicated that eggs are relatively low in the antineuritic vitamin. Recent work (13) has shown that egg white is devoid of vitamin B or the antineuritic factor but is rich in vitamin G (B_2), while egg yolk contains both factors (14).

As the result of work on the nutrition of tissue cultures, Heaton (15) concluded that factors associated with or identical with vitamins B and G are concerned in the multiplication of tissue cells. Not only was a thermolabile substance found necessary but a thermostable substance as well for the propagation of cells of chick embryo tissue. There was a difference in the response of different tissues. In addition, an inhibitory substance was found in yeast. These findings suggest that additional factors of a dietary nature may be concerned in hatchability.

Plan of Study

The general plans of the hen feeding experiments, together with results on hatchability and chemical composition, have been discussed in other papers (1, 2, 3). The vitamin studies on eggs have dealt with the effects on the eggs of a number of diets fed to hens under like environmental conditions. The birds were confined in long laying houses subdivided into pens, each of which was connected with a sunlit concrete floored runway. The work has extended over three years, in each of which new lots of birds were placed on experiment in September and fed until the following August.

The hen diets which have been compared fall into two groups.[1] The diets

[1] The diets used during the first year's work have been described by Titus, Byerly, and Ellis (2). Except for minor changes in the proportions of certain constituents, particularly as they affected the Ca:P ratio, and in diet numbers all the hen diets used in the present experiments are identical to or modifications of those just mentioned. Thus, the basal diet of Group A corresponds to diet 9, while the normal diet which contained the animal products corresponds to diet 8. Likewise, the basal diet of Group B corresponds to diet 10, the yeast 1 (B3) diet to diet 11, and the autoclaved yeast (B4) diet to diet 12. The other diets (A2, A3, B1, B2 and B3 with yeast 2) were introduced in the second or third year of the experiment.

in Group A contained a basal mixture of yellow corn, 500 parts; wheat bran, 245 parts; oatmeal, 150 parts; and alfalfa leaf meal, 55 parts; while those in Group B contained pearled hominy and desiccated meat meal in such proportion as to give 20 per cent protein in the mixture. The basal mixture in the first case was relatively rich in the vitamin B-complex, while in the second it was decidedly deficient. All diets were fortified with respect to vitamin A, vitamin D, and mineral deficiencies by the addition of 2 per cent of cod-liver oil and appropriate mineral substances.

In addition to the basal diets for each group, other diets were studied in which a portion of the basal mixture was replaced by supplements, either of a single feedstuff or of a combination of feeds. The list of diets was as follows:

Group A diets with a vitamin adequate basal mixture

Diet A—the basal mixture without supplement.

Diet A1—the normal diet. Contains 7 parts of fish meal, 8 parts of meat meal, and 5 parts of dried buttermilk to 80 parts of Diet A.

Diet A2—20 parts of rice bran to 100 parts of Diet A1.

Diet A3—20 parts of dried whey to 80 parts of Diet A.

Group B diets with the vitamin deficient hominy-meat meal base

Diet B—the deficient basal mixture.

Diet B1—15 parts of rice polish to 85 parts of Diet B.

Diet B2—15 parts of rice bran to 85 parts of Diet B.

Diet B3—15 parts of dry yeast to 85 parts of Diet B.

Diet B4—15 parts of autoclaved dry yeast to 85 parts of Diet B.

Dry yeast from two manufacturers was used in successive years and has been designated as yeast 1 and yeast 2. Yeast 1 was autoclaved for 4 hours at 15 pounds pressure for use in diet B4.

Fresh eggs produced during the period of maximum production, from February to July, were used in the rat feeding experiments. The rats were confined in individual cages equipped with 3-mesh screen bottoms. In the vitamin B and vitamin G experiments, the young rats were placed on the deficient diet at the age of 4 weeks. When further gains in weight had ceased after 7 to 14 days, therapeutic feeding of eggs and hen diets was initiated. These materials were fed on a 6 day per week basis for an 8 week period, at levels estimated to produce an average weekly gain of approximately 4 grams.

The vitamin A tests were made by the usual therapeutic method. The young rats were depleted of their vitamin A stores (as evidenced by cessation of growth) in 5 to 6 weeks. The eggs were fed 6 days per week for 8

weeks. Weekly weight records on gains in weight and food consumption were kept.

The studies on eggs were divided into 6 series as follows:

1. The growth response of rats to a test dose of whole egg produced on the normal diet and fed at the same level with different basal rat diets which varied in the presence and source of the vitamin B complex and the separate factors or groups of factors designated as vitamins B and G.
2. The distribution of the factors in the vitamin B complex in the yolks and whites of normal eggs.
3. The influence of hen diets from Groups A and B on the vitamin B complex in the whole egg.
4. The relationships between the vitamin B content of hen diets from Groups A and B and of the eggs produced by the hens.
5. The relationships between the vitamin G content of hen diets from Groups A and B and of the eggs produced.
6. The relative constancy and high potency of eggs in vitamin A produced on the basal diets of Groups A and B, supplemented with cod-liver oil but variable in the vitamin B complex and in pigmentation.

The Experiments

Series 1.—Eggs produced by hens on the normal diet (A1) were fed in test doses to measure the response of the rats on different test rations. The chief purpose of this study was the evaluation of methods for estimation of the vitamin B and vitamin G content of eggs, together with the possible detection of additional factors present in eggs.

Three basal rat diets used with or without modifications in the series of 7 rations were as follows:

Diet 147—Dried egg white, 20 per cent; dextrinized rice starch, 65 per cent; mineral mixture (16), 4 per cent; agar, 1 per cent; and lard, 10 per cent. Cod-liver oil was fed separately.

Diet 149—Like diet 147 except purified casein replaced the egg white.

Diet 191—Purified casein, 18 per cent; dextrinized rice starch, 48 per cent; salt mixture (16), 4 per cent; lard, 8 per cent; cod-liver oil, 2 per cent; and ground white corn, 20 per cent.

The seven comparisons fall into a number of groups according to vitamin deficiency. The vitamin B complex was measured in one case (I) where diet 149 was fed alone. Three diets contained sources of vitamin G in the form of (II) alkaline autoclaved yeast supplied at a 0.4 gram per day level, (III) egg white contained in diet 147, and (IV) neutral auto-

claved yeast supplied at a 0.3 gram per day level. All these diets were deficient in vitamin B, with possible differences in the supply of additional factors. Two other diets contained sources of vitamin B but were deficient in vitamin G. Rice polish extract (V) at a level which protected against polyneuritis but permitted only maintenance of weight was used in one case, and white corn (VI) contained in diet 191 in the other. In the last case (VII) rice polish extract as used in (V) and alkaline autoclaved yeast as used in (II) were both added to diet 149. Possibly this combination still left a deficiency in one or more of the newer factors associated with the vitamin B complex (17).

Averaged results on control rats on the several diets and dietary supplements are shown in Figure 1. The two autoclaved yeast preparations caused little, if any, difference in the response of the rats. The "alkaline" product was autoclaved at pH 9 for 2 hours while the neutral or untreated product was autoclaved for 4 hours. The pressure was 15 pounds in each case.

Rice polish (18) compared favorably with the extract of rice polish as judged by relative richness in vitamin B and lack of vitamin G. The extract was the concentrate from an extraction of rice polish with 85 per cent alcohol. Numerous tests have shown that the extract is a relatively pure source of the vitamin B fraction. Its addition to diet 149 at a level equivalent to 0.5 gram of rice polish permitted little more than maintenance of weight over extended periods of four to five months. During the third month, the rats developed the symptoms ascribed to vitamin G deficiency (17). The most noticeable effects were the skin disturbances, loss of hair, and general emaciation. The addition of autoclaved yeast relieved this condition and promoted a moderate rate of gain.

In keeping with the findings of Chick and Roscoe (13) egg white was found an excellent source of vitamin G with little contamination of vitamin B. A supplement of autoclaved yeast was without benefit but rice polish and wheat germ were effective (Figure 1) in the prevention of polyneuritis and the promotion of growth. No harmful effects such as those noted by Parsons (19) on high levels of egg white were observed on the level which was fed.

The use of white corn as a source of vitamin B (diet 191) was unsatisfactory owing to the irregularities in the behavior of the control rats. Certain ones showed the desired evidence of deficiency in vitamin G but others showed a rather abrupt change after approximately five weeks. From this point they gained slowly without interruption. This behavior may have been due in part at least to coprophagy.

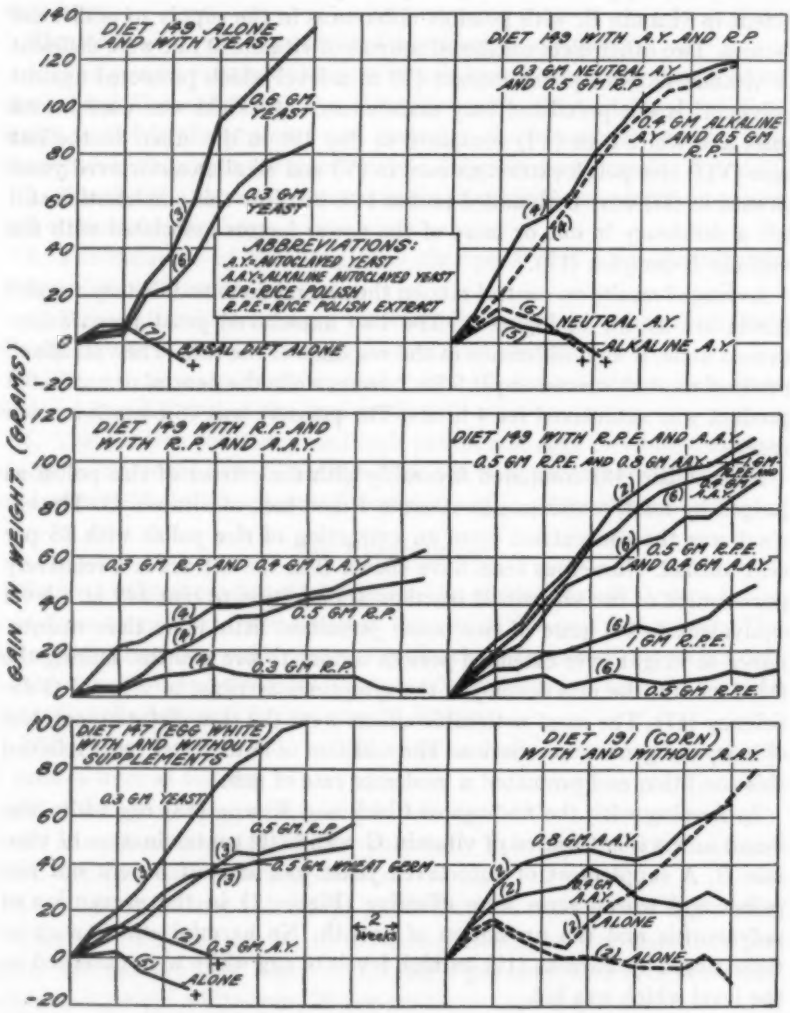
DIET 149 ALONE AND WITH YEAST
0.6 GM. YEAST
0.3 GM. YEAST
BASAL DIET ALONE
ABBREVIATIONS:
A.Y.=AUTOCLAVED YEAST
A.A.Y.=ALKALINE AUTOCLAVED YEAST
R.P.=RICE POLISH
R.P.E.=RICE POLISH EXTRACT
DIET 149 WITH A.Y. AND R.P.
0.3 GM NEUTRAL A.Y. AND 0.5 GM R.P.
0.4 GM ALKALINE A.Y AND 0.5 GM R.P.
NEUTRAL A.Y.
ALKALINE A.Y.
GAIN IN WEIGHT (GRAMS)
DIET 149 WITH R.P. AND WITH R.P. AND A.A.Y.
0.3 GM R.P. AND 0.4 GM A.A.Y.
0.5 GM R.P.
0.3 GM R.P.
DIET 149 WITH R.P.E. AND A.A.Y.
0.5 GM R.P.E. AND 0.8 GM A.A.Y.
0.5 GM R.P.E. AND 0.4 GM A.A.Y.
1 GM R.P.E.
0.5 GM R.P.E.
DIET 147 (EGG WHITE) WITH AND WITHOUT SUPPLEMENTS
0.3 GM YEAST
0.5 GM. R.P.
0.5 GM. WHEAT GERM
0.3 GM. A.Y.
ALONE
2 Weeks
DIET 131 (CORN) WITH AND WITHOUT A.A.Y.
0.8 GM. A.A.Y.
0.4 GM. A.A.Y.
ALONE
ALONE

FIG. 1.—Average gains or losses in weight of 97 rats used as controls in the experiments on the vitamin B-complex, the vitamin B and the vitamin G content of hen diets and eggs. The numbers in ( ) refer to the number of rats on which the averages are based. The average time of death is indicated by the symbol +. The supplemental feedings are all given on a daily basis.

Following a 10 to 14 day depletion period, groups of 6 rats evenly divided as to sex and distributed among the 7 dietary treatments so there was never more than 1 rat from a litter on a particular treatment were fed 2 cc. doses of the whole mixed egg daily for an 8 week period. The average results are shown in Table I. The lowest gain was made by Group I on the

TABLE I

RESULTS OF COMPARISON OF METHODS OF DETERMINING THE FACTORS IN THE VITAMIN B COMPLEX WITH A DAILY DOSE OF 2 CC. OF EGGS PRODUCED ON A NORMAL DIET

Rat diet and supplements fed	Ration deficient in	Number of rats	Initial weight on experiment	Eight week test period			
				Initial weight on egg	Total gain	Weekly gain	Daily feed
			gm.	gm.	gm.	gm.	gm.
(I) Diet 149 only.....	Complex	6	49	53	23	2.9	3.7
(II) Diet 149 with alkaline autoclaved yeast.....	B	6	47	65	34	4.3	4.1
(III) Diet 147 containing egg white....	B	6	47	61	32	4.0	3.6
(IV) Diet 149 with neutral autoclaved yeast.....	B	6	46	59	44	5.5	4.1
(V) Diet 149 with extract of rice polish.	G	6	49	60	42	5.3	4.9
(VI) Diet 191 containing white corn....	G	6	46	61	94	11.8	8.7
(VII) Diet 149 with sources of B and G	Neither B nor G	6	46	64	97	12.1	7.2

diet containing no vitamin B complex. Of the 3 groups on diets designed to measure the vitamin B content of the eggs, the one on egg white made the least and the one on neutral autoclaved yeast the greatest gain. The average gain of the rats on the neutral autoclaved yeast ration was 44 grams as compared to 32 and 34 grams respectively on the egg white and alkaline autoclaved yeast rations. The alkaline autoclaved yeast has appeared to be the most satisfactory supplement from the standpoint of the constancy of the product. It has been used throughout the work as a vitamin G supplement in determinations of the comparative vitamin B contents of the eggs produced on the various diets.

The two groups on vitamin G tests showed widely different behavior.

The behavior of control animals on diet 191 has been noted. In view of the growth attained by the controls, it was not surprising to find that 2 cc. of egg enabled the group of 6 rats to gain 94 grams in the 8 week period. This was comparable to that attained by the group (VII) which received both the autoclaved yeast and rice polish extract supplements. The 6 rats which received diet 149 plus the rice polish extract and 2 cc. of whole egg (Table I) gained an average of 42 grams in 8 weeks or slightly over 5 grams per week. The performance on this ration has appeared to warrant its use as a measure of the vitamin G content of the eggs.

Series 2.—Feeding tests on egg yolks from eggs produced on normal mixed diets were made with rat diets 147 and 149. The results of these tests are given in Table II. Twelve rats arranged in 2 groups received 1 cc. of yolk daily for 6 weeks, then for 4 weeks one group received a 0.5 gram daily supplement of rice polish rich in vitamin B and the other group 0.3 gram of autoclaved yeast as a source of vitamin G. The 1 cc. dose of egg yolk permitted an average weekly gain in weight of 1.9 grams during the first 6 weeks of the test. Rice polish did not materially increase the growth rate in the second period of 4 weeks, while autoclaved yeast did cause an increase from 1.5 to 5.0 grams per week. Thus, vitamin G appeared to be the limiting factor in the yolks.

Three groups of 3 animals each, shown in Table II, which received diet 149 with a daily dose of 2 cc. of yolk gained an average of 7.2 grams per week during an 8 week period. In a subsequent 2 week period, one group received the neutral autoclaved yeast, another the alkaline autoclaved yeast, and the third no additional supplement. Since the rats had passed through the cycle of most rapid growth by the time this period was reached, the weekly averages were low for all 3 groups. The autoclaved yeasts did not promote an increased rate of growth over the control group. However, the results again suggest that the neutral autoclaved yeast retained more of the vitamin B complex than the alkaline treated yeast.

Six rats received diet 147 which contained egg white as the source of vitamin G as well as protein. One group of 3 rats received 1 cc. and the second group of 3 received 2 cc. of egg yolk daily for 7 weeks, then for 3 weeks one rat in each group received rice polish, another neutral autoclaved yeast and the third alkaline autoclaved yeast. The average weekly gains for the 7 week period were slightly better than those on diet 149. The response to rice polish supplement during the subsequent 3 week period showed that vitamin B was the limiting factor on diet 147. Egg white, as shown by the results on diet 147 as well as from tests on the liquid

TABLE II

THE RELATIVE VITAMIN B AND VITAMIN G CONTENT OF EGG YOLK AS SHOWN BY THE GROWTH RESPONSE OF RATS ON BASAL DIETS WITH AND WITHOUT THE ADDITION OF SUPPLEMENTS RICH IN THESE VITAMINS

Feeding	Period I				Period II			
	Duration	Number of rats	Average		Supplement	Duration	Average	
			Weekly gain	Daily feed			Weekly gain	Daily feed
	days		gm.	gm.		days	gm.	gm.
Diet 149 with 1 cc. of egg yolk	42	6	2.3	5.2	.5 gm. rice polish	28	2.8	4.2
	42	6	1.5	5.5	.3 gm. aut. yeast	28	5.0	5.1
(Average)			1.9	5.4				
Diet 149 with 2 cc. of yolk	56	3	7.8	6.0	No addition	14	1.2	5.5
	56	3	7.3		.3 gm. aut. yeast	14	3.0	5.2
	56	3	6.5	—	.4 gm. alk. aut. yeast	14	2.0	5.5
(Average)			7.2					
Diet 147 with 1 cc. of yolk	49	1	4.3	5.3	.5 gm. rice polish	21	18.3	9.1
	49	1	3.0	4.9	.3 gm. aut. yeast		3.7	3.7
	49	1	1.3	5.3	.4 gm. alk. aut. yeast		1.0	3.8
(Average)			2.9	5.0				
Diet 147 with 2 cc. of yolk	49	1	5.7	5.5	.5 gm. rice polish	21	13.0	7.2
	49	1	8.7	6.4	.3 gm. aut. yeast		8.3	6.0
	49	1	9.0	5.3	.4 gm. alk. aut. yeast		2.7	5.8
(Average)			7.8	5.7				

TABLE III

THE VITAMIN B-COMPLEX IN WHOLE EGGS PRODUCED ON DIFFERENT DIETS, TOGETHER WITH THE RESPONSE OF THE RATS TO THE ADDITION OF VITAMIN B AND VITAMIN G-RICH SUPPLEMENTS. VALUES AS AVERAGE GAIN IN GRAMS PER WEEK

Feeding period	Dose of egg	Supplement to Diet 149	No. of rats	Group A: basal hen diet		Group B: deficient hen diet		
				A	A1 normal	B	B3 yeast 1	B4 aut. yeast 1
				gm.	gm.	gm.	gm.	gm.
First 4 weeks (a)*	2	None	4	7.5	4		1	−1
First 4 weeks (b)	8	None	4			4.5		
Second 4 weeks (a)	2	None	4	4.5	3			
Second 4 weeks (b)	8	None	4			8.3	12.7	11
Third 4 weeks (1a)	2	Rice polish	2	13.5	13.3			
Third 4 weeks (1b)	8	Rice polish	2			11.7	10.5	12.5
Third 4 weeks (2a)	2	Aut. yeast	2	9.0	6.3			
Third 4 weeks (2b)	8	Aut. yeast	2			8.5	15	10
First 4 weeks	4	None	4			2.3	3.7	1.8
Second 4 weeks	4	None	4			2.7	4.0	4.5
Third 4 weeks (1)	4	Rice polish	2			15.5	15.5	15.8
Third 4 weeks (2)	4	Aut. yeast	2			13	5.5	8.7

* Designations of groups of rats carried through the successive four week periods. The numbers 1 and 2 indicate division of "letter" group in the third period.

material, showed little or no vitamin B but moderate amounts of vitamin G. while the yolk appeared to be the richer in vitamin B.

Series 3.—Whole eggs produced on different diets were compared for the content of the vitamin B complex. The hen diets in the comparison consisted of the basal and the normal (A1) of Group A and the deficient mixture, the dry yeast 1 (B3) and the autoclaved dry yeast 1 (B4) of Group B. The eggs were fed daily in the liquid state to groups of 4 rats each. The experimental feeding was divided into 3 periods of 4 weeks each. In the third or final period the rats were paired within each group on added daily supplements of 0.3 gram of rice polish and 0.4 gram of alkaline autoclaved yeast.

The average weekly gains of the rats by 4 week periods on several doses of eggs from the hen diets are given in Table III. The 2 cc. dose of egg from the basal diet and the normal diet of Group A was sufficient for moderate growth. The former diet appeared to have produced eggs of the greater potency in the vitamin B complex. The hens on the deficient diet with the hominy-meat meal base produced eggs which were less potent than those from the adequate diets. Of the 2 groups on 4 and 8 cc. of eggs respectively, the latter produced gains in the rats approximately equal to 2 cc. of egg from the Group A diets. The grade of yeast added at a 15 per cent level to the deficient diet was clearly ineffective in increasing the vitamin B complex in the eggs. The groups on the 2 cc. dose of both the untreated and the autoclaved yeast failed to gain during the first 4 weeks of the test. An increase to 8 cc. in the second period which was continued through the third period permitted normal gains. The 4 cc. dose of these eggs was approximately equal to the 2 cc. dose of eggs from the normal diet. Subsequent tests on this grade of yeast showed that it possessed perhaps only 25 per cent of the potency of the yeast used for the rat feeding tests. The addition of rice polish and of autoclaved yeast in the third period showed that vitamin B was probably the limiting factor in whole eggs, but the supplements did not indicate any undue disproportion between vitamin B and vitamin G due to diet. However, the number of animals was too small to give a definite answer on this point.

Series 4.—The vitamin B content of a number of hen diets and of eggs produced on these diets was determined by rat feeding tests in Series 4. The rats were fed a vitamin B free diet which consisted of diet 149 supplemented with a 0.4 gram daily supplement of alkaline autoclaved yeast. The hen diets and the liquid eggs were then fed in test doses for an 8 week period.

TABLE IV

THE VITAMIN B CONTENT OF THE DIETS FED TO THE HENS AND OF THE WHOLE EGGS PRODUCED ON THESE DIETS AS MEASURED BY THE GAINS OF RATS ON A VITAMIN B-DEFICIENT RATION*

	Group A: basal hen diet			Group B: deficient hen diet					
	A	A1 normal	A2 rice bran	B	B1 rice polish	B2 rice bran	B3 yeast 1	B3 yeast 2	B4 aut yeast 1
Tests on hen diets:									
0.5 gm.† No. rats	3	8	3		4	4			
Gain‡ gms.	0	2.9	5.5		1.9	−.7			
1.0 gm: No. rat		4			4	10			
Gains, gms.		4.1			4.2	2.8			
5.0 gms: No. rat				6			5	4	4
Gain, gms.				died			died	died	died
Tests on eggs:									
1 cc.†: No. rats	7	7	7			6			
Gain, gms.	2.3	2.2	3.9			4.0			
2 cc: No. rats	6	17		4	6	6	4	7	4
Gain, gms.	7.3	5.3		2 died	5.9	7.3	died	3.9	2 died
3 cc: No. rats				4			4§	6	4
Gain, gms.				3 died			2.6	6.5	4.9
4 cc: No. rats				16			6		6§
Gain, gms.				5.5			3.4		3.0

* Rats on the vitamin B deficient ration die in 3 to 5 weeks.

† The daily dose fed to rats.

‡ The item of "Gain, gms." gives the average weekly gain for the 8 week test period.

§ One died, average on remainder.

The results of tests on the hen diets are given in Table IV. The table shows the characteristic components of the hen diets, the daily dose to the rat, and the number and the average weekly gains of the rats. The adequate diet of Group A, with or without special supplements, was fully 10 times as potent in vitamin B as the deficient mixture of Group B. Five grams was the maximum dosage of this diet which the rats consumed. The diets supplemented with 15 per cent of yeast also failed to protect the animals, although that designated as yeast 2 showed greater potency than yeast 1. Both rice polish and rice bran, incorporated as 15 per cent of the diet, were effective vitamin B supplements as evidenced by the approximate equality between the normal diet on the one hand and the deficient mixture supplemented with the rice by-products named.

The vitamin B tests on eggs from three years of experiments have been summarized in Table IV. Only the average weekly gains of the groups of rats on various doses of whole eggs are given. A total of 127 rats, exclusive of controls, have been fed eggs produced on 9 diets at levels ranging from 1 cc. to 4 cc. daily for 8 weeks. The normal diet (A1) and the deficient hominy-meat meal diet (the basal of Group B) have received the greatest attention. Seventeen rats on a 2 cc. dose of the former and 16 rats on a 4 cc. egg dose of the latter produced approximately equal growth.

Although relatively small lots of rats ranging in most cases from 4 to 7 were used in the other comparisons, two and sometimes three levels of egg were fed in the usual case. Thus, the effect of a particular hen diet or kind of feed can be measured by the results as a whole from several lots of rats.

The eggs from the normal diet were possibly less potent than from the Group A basal diet without the animal proteins or the normal diet with 16.7 per cent of rice bran. One cc. of egg from the last named diet was sufficient to produce an average gain of 3.9 grams per week. Eggs from hens on the Group A basal diet, fed at 1 cc. and 2 cc. levels, produced gains of 2.3 and 7.3 grams per week respectively, as compared to 2.2 and 5.3 grams on 1 cc. and 2 cc. of eggs from the normal diet. The ability of rice bran and rice polish to raise the vitamin B content of the eggs was also shown when these feeds were added to the deficient mixture in Group B. Yeast 1 failed to materially increase the potency of the eggs although it did furnish some protection against polyneuritis in the hens. The autoclaved yeast did not alter the general order of results. Yeast 2 was reasonably satisfactory as a supplement although not as good as rice bran.

Series 5.—This series of tests on the vitamin G content of hen diets and of eggs was analogous in the plan of work to Series 4. The list of diets

differed in the omission of the autoclaved yeast diet and the substitution of the dried whey diet (A3) for the basal diet of Group A. The vitamin G deficient ration fed to the rats consisted of diet 149 with the addition of sufficient extract of rice polish to protect against polyneuritis and permit maintenance of weight.

The data on the vitamin G tests of the hen diets are summarized in Table V. The 0.5 gram dose of the diets based on the vitamin-adequate

TABLE V

THE VITAMIN G CONTENT OF DIETS FED TO HENS AND OF THE EGGS PRODUCED ON THESE DIETS AS MEASURED BY THE GAINS OF RATS ON A VITAMIN G-DEFICIENT RATION*

Item	Group A—basal hen diet			Group B—basal hen diet			
	A1 normal	A2 rice bran	A3 dried whey	B1 basal	B2 rice bran	B3	
						yeast 1	yeast 2
Tests on hen diets							
Daily dose, gms....	0.5	0.5	0.5	5.0	1.0		2.0
Number of rats†...	6	6	5	5	5		5
Weekly gain, gms..	3.6	4.3	4.9	1.9	4.1		5.0
Tests on eggs							
Daily dose, cc.....	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Number of rats‡...	12	6	6	12	12	12	6
Weekly gain, gms..	4.9	7.9	6.5	4.1	4.5	3.8	6.0

* Rats on the vitamin G deficient ration alone gained 0.9 grams per week.

† Female rats.

‡ Males and females equally distributed.

mixture was sufficient in all cases to promote weekly gains of 3 to 5 grams. The diet containing 20 per cent of dried whey in place of the combined animal product supplement of the normal diet produced the best gains for the Group A diets. A ten-fold increase in dose was required of the basal diet of Group B for a gain of approximately 2 grams per week. The inclusion of 15 per cent of rice bran or yeast 2 in diets with the deficient mixture of Group B as the base, raised the vitamin G content to a level which made 1 gram of diet of the first and 2 grams of the second named supplement approximately equal to 0.5 gram of the Group A diets. The differences in the diets in vitamin G content were possibly not as pronounced as in vitamin B. However, the 5 gram dose of the deficient diet in Group B approximated a full feed for the rats since they consumed only 0.8 gram of diet 149 per day in addition to the 5 gram dose. Yeast 2 appeared relatively

richer and the rice bran relatively poorer in vitamin G than in vitamin B when fed as components of the hen diets.

All determinations on the vitamin G content of the eggs were made with a 2 cc. dose of egg. Comparisons were made of 5 diets in one year and of 6 diets in another year. Three diets were the same for both years while in a fourth diet yeast 2 replaced yeast 1. Results on the repeated diets showed differences which ranged from 0.1 to 0.4 gram in average weekly gains of the groups of rats. Accordingly, the groups from both years have been combined. The results are included in Table V.

The normal diet, the deficient diet alone and with yeast 1 or rice bran supplement apparently produced no marked differences in the vitamin G content of the eggs. The average weekly gains of 12 rats in each case were 4.9, 4.1, 3.8, and 4.5 grams respectively. Six rats on eggs from the rice polish diet (not included in the Table) gained equally well. With the wide difference noted in the vitamin G content of the hen diets as well as in the vitamin B content of the eggs, the lack of difference in the vitamin G content of the eggs from the diets named is noteworthy. The use of dried whey in place of the combination of animal protein supplements in the normal diet and the addition of rice bran to the normal diet resulted in an increased gain in the rats on egg tests. Paired comparisons were possible in a number of cases where rats of the same litter and sex were used on different eggs. All 6 rats on eggs produced on the rice bran-normal diet gained more than their mates on the same diet without the rice bran and on the deficient diet. Dried whey was no different from the mixed animal protein supplement in its effect on the eggs. In 5 of 6 cases the rats on eggs from the yeast 2 diet gained more than their mates on the deficient diet.

The results as a whole suggest that the hen diets did not affect the vitamin G content of the eggs to the extent which has been noted for vitamin B. Lack of vitamin G in the diet apparently did not result in any pronounced drop in the vitamin content of the egg. On the other hand, the inclusion of foods rich in vitamin G in the normal diet used in these experiments tends to enhance the vitamin G content of eggs.

The Vitamin A Content of the Eggs

Comparisons of the vitamin A content of eggs produced on the deficient diet with that of eggs produced on the normal diet were made in 1930 and again in 1931. These eggs were chosen because of their wide difference in pigmentation as well as other differences due to diet. The hominy-meat meal diet produced eggs with yolks nearly devoid of yellow pigments,

while the normal diet produced characteristic highly pigmented yolks. Rats which had become depleted of their vitamin A stores as manifested by cessation of growth were given daily feedings of yolk in the 1930 tests. Doses of 0.2 and 0.4 cc. were fed. The averaged gains in weight of the groups of rats during the 8 week period totaled 60 and 62 grams respectively on 0.2 cc. of egg yolk from the deficient and the normal diet. In 1931, the yolk and white were mixed and fed at a 0.1 cc. level. Twelve rats which had gained an average of 80 grams during a depletion period ranging from 37 to 40 days were paired according to sex (3 pairs of each sex), litter and weight. One of each pair was fed daily 0.1 cc. of egg from the deficient diet and the other an equal amount of egg from the normal diet. Numerous cases of xeropthalmia were cured during the early part of the period and at the close of the 8 week test period all animals appeared normal in health. The average gain was 47 grams for the deficient and 32 grams for the normal group. Considered by pairs, the rats in the former group gained more in every case than their mates in the latter group. These results indicate that the diet deficient in vitamin B complex produced eggs with a greater vitamin A potency although greatly lessened pigmentation.

The ability of the hens on the former diet, deficient in vitamin B complex, to store more vitamin A in the eggs than those on normal diet was probably due to a difference in the rate of egg production. The 2 per cent of cod-liver oil which was added to both diets was adequate for storage of a generous supply of vitamin A regardless of the amount naturally contained in the other foodstuffs of the diets.

From the data of Murphy and Jones (20), as well as those of the present study, it appears that 0.1 cc. was more than sufficient to supply 1 unit of vitamin A. On this basis, a 50 gram egg, exclusive of shell, produced on either of the diets studied, supplied over 500 units of vitamin A.

The importance of these results as they relate to hatchability and other factors concerned in the present problem rests in the fact that a simple diet of hominy, meat scrap, minerals, and cod-liver oil produced eggs which were nearly colorless yet adequately supplied with vitamin A. Therefore, vitamin A and probably vitamin D were not concerned in the low hatchability of eggs on the deficient diets studied.

Discussion

In view of the apparent multiplicity of factors in the vitamin B complex, any comparative studies on the content of a foodstuff in vitamin B or vitamin G as at present designated raises the question of the balance and

distribution of the factors in the test diet used for the measurements. The results from the first series of experiments on the growth response of rats fed a 2 cc. dose of egg along with different basal diets and vitamin supplements while they showed a satisfactory basis for the estimation of vitamin B and vitamin G, disclosed little evidence of the presence of other factors of the vitamin B complex. Subsequent work may indicate the significance of certain of the differences among the seven dietary treatments. The rice polish extract presumably contains, in addition to the B_1 factor, a B_3 factor necessary for pigeons (21) but not needed by rats or chickens (4). It is possible that yeast autoclaved in an alkaline medium may not retain the B_4 factor (22) which is required by rats. Apparently, egg white left the same deficiency as the alkaline autoclaved yeast. While the growth made by the rats on the combination diet of rice polish extract and alkaline autoclaved yeast was enhanced by the feeding of the 2 cc. dose of egg, it is not certain that the difference between the control and the egg supplemented groups was due to the B_4 and other unrecognized factors or to added supplies of the B_1 and B_3 factors.

While it had been shown previously, as noted, that whole eggs contained both vitamin B and vitamin G, and that egg white was lacking in vitamin B, the experiments in Series II on normal eggs showed that the yolks were relatively richer in vitamin B but that the whole eggs were somewhat richer in vitamin G.

When measured for the relative content of the vitamin B complex in Series 3, the eggs produced on different diets from Groups A and B showed an order of potency comparable to that found in subsequent measurements of vitamin B. The responses of the rats in these tests on the vitamin B complex when rice polish or autoclaved yeast were added to the ration during the third period of the experiment are indicative of the usual observation that whole eggs are richest in vitamin G.

Diet may equalize or even reverse this relationship in the vitamin B and vitamin G content of eggs since a pronounced variation may occur in the former and only a small variation in the latter. Such supplements as rice bran and rice polish rich in vitamin B, when added to hen diets promoted the production of eggs relatively rich in this vitamin but generally with little increase in vitamin G. On the other hand, eggs produced on the deficient diet alone showed approximately half the vitamin B content of normal eggs.

While pronounced differences existed in the potency of the hen diets in both vitamin B and vitamin G, the vitamin G content of the eggs was

apparently not affected to the same extent as the vitamin B content. The observations on the hens and on the hatchability of the eggs are of particular interest in this connection. The hens on the deficient diet of hominy, meat meal, minerals, and cod-liver oil showed a high incidence of polyneuritis during the first four months on experiment.

The birds also laid relatively few eggs, presumably as a result of the dietary. In the late winter and early spring during the usual seasonal period of maximum production, the hens were greatly improved in health. Egg production rose sharply in March of each year but the hatchability remained at a low level since the average for three years was approximately 15 per cent.

Perhaps the most important point in the results from the use of supplements in the deficient ration was the richness of the rice bran and rice polish in vitamin B as compared to yeast. These products were more potent than either of the yeast preparations used in the hen diets. Yet yeast 1 afforded a noticeable benefit to the hens although it did not affect the vitamin B content of the eggs. Yeast 2 was intermediate in effectiveness to yeast 1 and the rice by-products. Both rice bran and rice polish afforded excellent protection to the health of the hens and increased the vitamin B content of the eggs to a level equal to that on normal diets.

While the 15 per cent of rice bran appeared equal, if not superior, to an equal amount of yeast 2 as a vitamin G supplement to the group B basal diet in the rat tests, yeast 2 nevertheless was the more effective of the two as judged by the potency of the eggs.

Apparently the diets of Group A were equally good for the production of eggs of a reasonably high percentage hatchability (3). The vitamin G content of the eggs produced on these diets was not unlike that produced on the Group B diets. Likewise certain of the Group B diets supplemented with feeds especially rich in vitamin B produced eggs equal to those of the Group A diets in vitamin B.

The experiments do not permit of definite conclusions regarding the relation of vitamin B and vitamin G to hatchability. The incidence of polyneuritis among the baby chicks from eggs produced on the Group B basal diet indicated the necessity for this factor. The failure to secure any improvement in hatchability when the yeast or the rice by-products were added to the deficient diet showed that other factors were involved.

While the diet of the hen did not produce especially wide differences in either the vitamin B or the vitamin G content of the eggs, the variations due to the first named factor were nevertheless noteworthy. The lack of

difference in the vitamin G content of the eggs may have been due in part to the relatively low vitamin G content of most, if not all, of the diets.

The richness of eggs in vitamin A was manifested in the results of the 1930–31 experiments. Undoubtedly, the cod-liver oil added to the diets enhanced the vitamin A content of the eggs produced on the normal diets. The deficient diet of Group B yielded eggs richer in vitamin A than generally produced under average conditions although they appeared nearly devoid of yellow pigment.

Summary

Two groups of poultry diets, one containing a basal mixture moderately rich and the other a basal mixture deficient in vitamin B and vitamin G which had been found to affect egg production and hatchability, were studied for the effects of the diet of the hen on the vitamin B and vitamin G content of the eggs.

Egg yolks produced on diets adequate in the vitamin B complex contained relatively more vitamin B than vitamin G, while the whole egg was relatively less potent in vitamin B than in vitamin G.

Variations in the vitamin B content of the eggs were produced by the diets fed the hens. Eggs produced on a basal diet of hominy, meat meal, minerals, and cod-liver oil contained from one-third to one-half as much vitamin B as those produced on the same basal diet with rice bran or rice polish incorporated into the mixture or on normal diets containing ground whole cereal grains and mill feeds, with or without protein supplements.

The variations in the vitamin G content were less pronounced. A 2 cc. dose of egg produced weekly gains in rats of 3.8 to 7.9 grams. Although none of the diets was unusually rich in vitamin G, the lack of difference in the eggs was noteworthy in view of the wide difference between the Group A and B diets. In certain cases the vitamin B content was equal to or in excess of the vitamin G content.

The vitamin A content of the eggs was maintained at a high level by the addition of cod-liver oil to both groups of diets, although the basal diet of Group B produced eggs which were low in vitamin B and strikingly deficient in the yellow pigments usually associated with vitamin A.

In the diets studied, the variation or lack of variation in those vitamins determined apparently bore little relation to the hatchability of the eggs.

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COMPARATIVE EFFECT OF TOMATO AND ORANGE JUICES ON URINARY ACIDITY

By

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IN A previous paper (5) the effect of fresh Malaga grapes, several kinds of grape juice, grape concentrate, and Thompson seedless and Muscat seeded raisins on the urinary acidity was reported. In the experiments with grapes and grape products a 1000 cubic centimeter portion of grape juice or its approximate equivalent of 300 grams of concentrate or raisins was employed. In a subsequent paper (6) the effect of figs and a smaller amount of raisins is given. All of these materials exerted a marked effect on the urinary acidity. Because of their present general use in the diet, it also seemed desirable to ascertain the effect of daily ingestion of similar amounts of tomato and orange juices.

Blatherwick (2) reported that tomatoes produced significant changes in the urine, the pH being increased (decrease of H^+) and the ammonia excretion being decreased. His data show that the maximum pH change would be about 0.3 pH unit. In his experiments 200 and 300 grams of tomatoes daily were added to the basal ration. One subject was used to test the effectiveness of each quantity of tomatoes. Apparently fresh whole tomatoes were used in his experiments.

Blatherwick and Long (3) have shown that increasingly large amounts of orange juice produce an increase in the pH of the urine with a decrease in the ammonia content. They used 600 cubic centimeters of juice the first day, increasing the total daily consumption by 600 cubic centimeters each day so that on the fourth or last day 2400 cubic centimeters of juice were taken by each of the two subjects. The pH changes effected by the orange juice were about 0.6 pH unit.

The experiments with the tomatoes (2) did not cause very marked changes in the pH of the urine, nor did they answer the question of the effect of larger quantities of tomato juice of the type such as is now commercially available for table use. Also the orange juice experiments (3) did not show the effect produced by the daily ingestion of some given constant quantity of juice.

The present experiments were of twelve days' duration. Each experiment was divided into two consecutive periods. For the first five days each person received only the basal ration. During the next seven days each received, in addition, 1000 cubic centimeters of either tomato juice or orange juice. Three normal young men subjects were employed in each experiment.

The basal diet was the same as that described in a previous report (5). The 1000 cubic centimeter portions of the juices were used in order that the resulting data would be comparable to those obtained in the previous studies in which 1000 cubic centimeter portions of grape juices were used. A standard commercial tomato juice and the juice from fresh Valencia oranges were employed.

All analytical determinations were made in duplicate or triplicate, the average of closely agreeing duplicates only being reported. The methods were the same as those described in a previous study (5). In addition to the urine analyses, determinations of the ash content, the alkalinity of the soluble ash, the alkalinity of the insoluble ash, and the organic acid content were made upon composite samples of the tomato and orange juices.

Results and Discussion

As is shown in Tables I and II, the effect of ingestion of the tomato and orange juices on the pH of the urine is very marked. On the basal diet the pH generally was at equilibrium at 5.80 to 5.70. However, the pH in two instances was lower, being 5.55 for subject R (Table II) and 5.40 for subject N (Table I). The average range compares well with the corresponding range observed in previous experiments (5, 6).

With both the tomato and orange juices there was a generally uniform increase of pH of the urine following their addition to the diet. This increase was such that the pH during the last three days of the experiment was from 0.95 to 1.3 pH units higher than that of the fourth or fifth day of the experiment. The tomato juice produced an average change of 1.2 pH units, while the orange juice caused a change of about 1.05 pH units.

The present results with tomato juice are rather striking when compared to those obtained by Blatherwick (2) with 200 and 300 gram portions of tomatoes. Whereas his maximum pH change of the urine was approximately 0.3 pH unit, the average change in the present experiments was four times as great. This difference very possibly may be largely the result of the ingestion of three to five times the quantity of tomato material. This relation will be discussed in a later paper of this series. Ap-

TABLE I
EFFECT OF TOMATO JUICE ON COMPOSITION OF URINE

Day*	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Ammonia N	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Ammonia N	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Ammonia N
	Subject K					Subject N					Subject O				
	cc.		cc.	cc.	mg.	cc.		cc.	cc.	mg.	cc.		cc.	cc.	mg.
1	1320	6.05	678	43	154	760	5.60	512	175	91	1100	6.30	800	155	140
2	1460	6.05	612	87	139	690	5.45	590	218	99	1190	6.25	681	152	140
3	1100	5.90	540	133	121	580	5.40	509	188	104	890	5.70	587	157	151
4	1110	5.70	525	102	166	720	5.45	540	196	122	980	5.80	510	143	135
5	1160	5.80	544	127	167	840	5.40	590	223	140	740	5.80	523	158	146
6	2060	6.20	705	58	153	1430	6.10	626	186	143	940	5.95	538	55	113
7	1750	6.60	699	56	135	1420	6.20	780	131	128	1590	6.40	652	38	127
8	1820	6.50	625	55	127	1250	6.25	620	63	100	1730	6.50	769	27	123
9	1530	6.70	750	50	106	1180	6.30	635	78	118	1470	6.55	721	27	120
10	1630	6.60	740	55	101	1150	6.50	632	42	92	2300	6.85	896	26	111
11	1900	6.75	788	58	82	1300	6.70	635	43	75	1980	6.80	823	31	108
12	1970	6.70	756	49	66	1690	6.70	788	30	58	1850	7.10†	711	29	95

* Days, 1–5 inclusive, basal diet.
Days, 6–12 inclusive, basal + 1000 cc. tomato juice.
† Denotes basic urine.

TABLE II
EFFECT OF ORANGE JUICE ON COMPOSITION OF URINE

Day*	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Ammonia N	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Ammonia N	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Ammonia N
	Subject P					Subject Q					Subject R				
	cc.		cc.	cc.	mg.	cc.		cc.	cc.	mg.	cc.		cc.	cc.	mg.
1	620	6.00	756	108	191	1060	6.30	571	142	131	670	5.75	537	239	206
2	770	6.10	730	165	191	980	6.35	586	135	128	550	5.55	504	242	243
3	690	5.60	702	182	195	820	5.85	535	128	143	590	5.50	520	235	177
4	900	5.75	635	105	179	1380	5.85	502	130	152	750	5.60	519	232	210
5	850	5.70	683	99	180	1160	5.85	625	117	136	880	5.55	670	218	185
6	1260	5.90	739	106	141	1110	5.80	881	109	116	680	5.70	581	114	131
7	2080	6.35	782	74	108	2050	6.50	817	81	140	1460	6.40	784	83	111
8	1220	6.50	760	75	105	1850	6.60	847	75	132	1220	6.30	790	92	125
9	1090	6.50	856	72	113	1410	6.60	809	65	125	1210	6.30	815	97	130
10	1330	6.65	929	79	105	1660	6.75	823	60	116	790	6.35	711	87	125
11	1950	6.65	871	57	85	1420	6.75	836	60	106	760	6.50	737	88	110
12	1730	6.90	761	41	62	1800	6.80	842	61	102	960	6.50	727	82	105

* Days, 1–5 inclusive, basal diet.
Days, 6–12 inclusive, basal diet + 1000 cc. orange juice.

parently commercially packed tomato juice such as used in these experiments is as effective in increasing the urinary pH as are comparable quantities of grapes, grape products, figs, and orange juice.

A comparison of the present results with orange juice and those of Blatherwick and Long (3) is also of particular interest. With a fourfold increase of orange juice daily ingested during four consecutive days, their data show an average final increase of urinary pH of only 0.5 to 0.6 pH unit, a part of this increase occurring each day. However, the present studies indicate that with a constant daily ration of orange juice the maximum increase was not obtained until the fifth day, the increase then being about 1.05 pH units. Since the 1000 cubic centimeter portions of juice used in the present studies were less than even the 1200 cubic centimeter portions of the second day of Blatherwick and Long's studies, the question may be raised as to what would be the maximum effect produced by the 2400 cubic centimeter portions used by Blatherwick and Long if their experiments had been continued for two or three days longer.

Blatherwick and Long (3) have concluded that the drinking of large amounts of orange juice resulted in the production of alkaline urines. This is obviously not generally or always true. In the present studies an average total increase of urinary pH of 1.05 above that of the basal diet was observed, yet alkaline urines were not obtained. On the contrary, the above workers reported a total pH change of the urine of 0.5 or 0.6 unit above that of the basal diet, or approximately half of that obtained in the present studies. Yet they secured alkaline urines. It therefore seems apparent that the production of alkaline urines in such experiments is not a specific attribute of orange juice. Rather, the production of such alkaline urines is dependent upon the fact that the urinary pH on the basal diet alone was only 0.1 to 0.2 or 0.3 pH unit below neutrality. In such a case any increase, even as small as 0.3 or 0.4 pH unit would, of course, produce the alkaline urines. Consequently, the value of orange juice in lowering urinary acidity may be considerably greater than was indicated by the 0.5 to 0.6 pH unit lowering secured by Blatherwick and Long. That is, subjects with very acid urines may also secure very marked decreases of such acidity (or increase of urinary pH).

The slightly greater increase of urinary pH produced by the tomato juice in comparison to that produced by orange juice is noteworthy when the alkalinities of the ash are considered. While the tomato juice produced the greater change of pH, the ash of the orange juice nevertheless possessed the greater total alkalinity. The total alkalinity of the ash of the tomato

juice was equivalent to 57.2 cubic centimeters of one-tenth normal hydrochloric acid per 100 grams of juice, while that of the orange juice was equivalent to 78.2 cubic centimeters of one-tenth normal hydrochloric acid per 100 grams of juice. However, the alkalinity of the water soluble ash of the tomato juice was equivalent to 47.2 cubic centimeters of one-tenth normal acid, while the corresponding alkalinity of the orange juice was equivalent to 50.75 cubic centimeters of one-tenth normal acid. That is, about 82.5 per cent of the total alkalinity of the tomato juice ash is that of the water soluble ash, while the soluble alkalinity of the orange juice ash is only about 65 per cent of the total alkalinity of the ash. The ratios of soluble ash alkalinity to insoluble ash alkalinity are 4.7 to 1 and 1.85 to 1, respectively, for tomato and orange juices. It is, therefore, possible that not only the magnitude of the total alkalinity but also the ratio of the alkalinities of the water soluble ash and water insoluble ash is important in determining the effect on the reaction of the urine. If such a condition does prevail, it appears that the alkalinity of the soluble ash is more effective in increasing the pH of the urine.

From the data in Tables I and II showing the values for total acids and ammonia excreted daily, it is evident that there was a considerable decrease in their concentrations after both the tomato and orange juices were added to the basal diet.

The evaluation of the quantitative relationship between the carbon dioxide binding power of the blood plasma and the excretion of acid in excess of fixed bases, as measured by determining the ammonia and titratable acid, has been developed by Fitz and Van Slyke (4). In a previous study with grapes and grape products (5) this method of estimating the lowering of the alkaline reserve was employed. It was shown that the basal diet alone lowered the alkaline reserve and the grapes and grape products assisted in increasing the alkaline reserve to a point above its previous level. From a comparison of the data in Tables I and II with those of the previous study, it is evident that both tomato and orange juices increased the alkaline reserve of an individual even above the normal as estimated from the data of the first day of an experiment. This was indicated approximately by the considerably smaller values for the ammonia and titratable acidity titrations. The raising of the alkaline reserve was of the same order of magnitude, or slightly greater, as was that produced by equivalent quantities of grapes and grape products.

The relation between the percentage oxidation of the organic acids and the changes of the pH are of interest. The organic acid content of the to-

mato and orange juices was determined by the method of Van Slyke and Palmer (7). The total amounts daily ingested (as cubic centimeters of tenth-normal acid) are given in Table III. The differences of the average daily organic acid titration of the urine with and without the juices added to the basal diet are also given. From these values the percentage of the organic acids oxidized in the body may be computed. An average of 90.7 per cent of the organic acids of the tomato juice was oxidized while an average of 93.8 per cent of the organic acids of the orange juice was oxi-

TABLE III
ORGANIC ACIDS INGESTED AND OXIDIZED
(As cc. N/10 HCl)

	Tomato Juice			Orange Juice		
	K	N	O	P	Q	R
Average daily organic acid titration for basal diet period	580	548	600	701	564	550
Average daily organic acid titration for basal diet+tomato and orange juice	723	673	733	814	836	735
Difference	143	125	133	113	272	185
Organic acids ingested daily from tomato or orange juice	1450	1450	1450	3087	3087	3087
Per cent oxidation of organic acids	90.1	91.1	90.8	96.3	91.2	94.0

dized. However, the tomato juice produced a somewhat greater change of urinary pH. If the organic acids do influence the pH, as is possible, this difference of effect may be in part the result of the higher organic acid content of the oranges. The data in Table III indicate that the organic acid content of the orange juice used in these tests was slightly more than twice as great as that of tomato juice. Hence the final average amount of organic acids left unoxidized from the orange juice may be somewhat larger than that from the tomato juice. Using the data in Table III it appears that, expressed as cubic centimeters of tenth-normal acid, about 135 parts of the organic acids of tomato juice and 190 parts of the organic acids of orange juice are not oxidized. It is therefore probable that the unoxidized organic acid residue is a limiting factor in relation to the magnitude of the pH change of the urine in such experiments.

The present value of 93.8 per cent oxidation of organic acids of orange juice compares well with that of 94 per cent reported by Blatherwick and Long (3) for orange juice.

SUMMARY

Experiments with men subjects on a basal diet and on the same basal supplemented by tomato and orange juices are reported. The following results were observed when the juices were added to the basal ration.

1. An average increase of the urinary pH of 1.20 pH units was produced by 1000 cubic centimeters of tomato juice taken daily. An equal quantity of orange juice produced a similar average increase of 1.05 pH units.

2. Corresponding decreases in both the ammonia excreted and in the total acidity were noted. The average changes produced by the two juices were approximately the same.

3. There was an increase of the alkaline reserve, calculated according to the method of Fitz and Van Slyke, above the normal for each subject. This increase was quite marked for both juices.

4. There appeared to be a correlation between the alkalinity of the ash and the reaction of the urine. A more basic reaction was associated with the higher ratio of soluble alkalinity to insoluble alkalinity of the ash. The tomato juice exhibited the higher ratio and produced a somewhat larger change in reaction.

5. An increase occurred in the organic acids excreted when either tomato or orange juice was added to the basal ration.

6. The average oxidation of the organic acids of tomato juice was 90.7 per cent while that of orange juice was 93.8 per cent.

The writers wish to express their appreciation of the interest and advice of Dr. W. V. Cruess, upon whose suggestion this study was initiated. The full coöperation of the men taking a part in the diets is gratefully acknowledged.

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THE EVALUATION OF THE PHOSPHORUS DEFICIENCY OF THE RICKETS-PRODUCING DIET

By

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WE were eager to evaluate the extent of the deficiency in the rickets-producing diet of Steenbock and Black no. 2965 (1). This contains an excess of calcium and a deficiency of phosphorus. That phosphorus intake may be a limiting factor in growth has been shown by Osborne and Mendel (2). Upon a diet of 18 per cent of either edestin or casein the growth of rats was inhibited, but resumed when phosphate was added. They cite this as an example of Liebig's law of the minimum. Their experiments antedated the discovery of vitamin D; but the diets contained 18 per cent of butter and therefore must have included considerable vitamin. The contrary has been reported by Bethke, Steenbock and Nelson (3) who state[1] that "with casein as the protein, fed at a level of 18 per cent of the ration, the phosphorus cannot be sufficiently reduced to cause nutritive failure. Even with lower levels of cod liver oil intake, increase of phosphate does not lead to increased growth."

It is well known that the calcium and phosphorus metabolism are interdependent. An increase in the calcium may cause a relative insufficiency of the phosphorus. Examination of the calcium and phosphorus content of the diets given in Table I shows that the phosphorus of the diet of Bethke, Steenbock and Nelson lies between that of the edestin and casein diets of Osborne and Mendel and that the calcium is essentially the same. In the rickets-producing diet No. 2965, the phosphorus which is considered below the optimal for rats is higher than in the low phosphorus diets, but the excessive calcium makes it relatively low.

Rats fed the rachitogenic diet No. 2965 of Steenbock and Black, which contains a moderate amount of vitamin A and is deficient in D, gain in weight for only 3 to 4 weeks. After that they decline in weight and die in

[1] Reference 3, page 283.

TABLE I

Author	Diet	Calcium	Phosphorus	Ca:P
		mg. per cent	mg. per cent	
Osborne and Mendel (2)	Edestin 1	500	35	14
Osborne and Mendel (2)	Casein 2	500	169	3
Osborne and Mendel (2)	Control 1	546	372	1.4
Osborne and Mendel (2)	Control 2	546	511	1
Bethke, Steenbock, and Nelson (3)	Low phosphorus	447	55	8
Steenbock and Black (1)	No. 2965	1250	250	5

9 to 12 weeks. Hence such animals are unsuitable for investigation of the effect of phosphorus deficiency. The addition of cod liver oil to the diet prevents the development of rickets or cures it. Bethke, Steenbock, and Nelson (3) have shown the interrelationship of calcium-phosphorus imbalance and vitamin intake on growth. With increase of vitamin, the growth is improved for a given calcium deficient diet. Thus we hoped without changing the mineral content of the diet to maintain the animals in good condition and growth for a long time.

In addition to the growth, other criteria of metabolism are available: x-ray pictures, the analysis of the blood serum, bones, and tissues, and the measurement of the intake and output of calcium and phosphorus.

When rats are fed a high calcium low phosphorus diet such as No. 2965 of Steenbock and Black, whether or not vitamin D is also fed, a relatively high calcium low phosphorus retention results, though both retentions are below normal. These findings are based upon short-time studies (1 to 5 weeks) (4, 5). The question at once arises as to what the ultimate outcome of such a regimen would be. Does the type of retention change so that the calcium-phosphorus balances no longer reflect the proportions present in the food, or does the body composition, especially that of the bones, become altered?

EXPERIMENTS

Animals were bred from the Babies and Childrens Hospital stock colony of albino rats of Wistar Institute stock. The mothers were fed Sherman's diet B (6) consisting of whole wheat $\frac{2}{3}$ and whole milk powder $\frac{1}{3}$ plus 1.33 per cent NaCl. In addition they were given meat and liver, about 5 gm. each, and lettuce once a week. While pregnant they were also given 400 mg. of compressed yeast tablet daily.

The young were weaned at 21 days and continued on Sherman's diet without any additions for seven days. At the age of 28 days they were placed upon the experimental diet. This consisted of Steenbock and Black's diet No. 2965—76 per cent ground yellow corn, 20 per cent gluten flour, 3 per cent $CaCO_3$ and 1 per cent NaCl. To each 100 gm. of diet 2 gm. of cod liver oil were added. The diet was made fresh every week. The experiment was run in 1930 and repeated in 1931. Metabolism studies were made for 7-day periods, during the 5th, 6th, and 7th weeks the first year and 9th, 14th, and 19th weeks the second year. It was feared that the animals in the metabolism cages would not be strictly comparable in growth to those with easier access to food and more ample room for exercise, provided by our stock cages. Therefore in the first series the metabolism was studied for only 3 weeks. For the remaining 17 weeks they were kept in the screened stock cages without bedding. In the second series the control and experimental animals were confined to the metabolism cages throughout the experimental period and were controlled by litter mates in stock cages on both the experimental and standard diets.

The metabolism cages which caused separation of urine and feces were cleaned daily with distilled water and a rubber policeman. The food was analyzed for calcium and phosphorus and, from the weight of that consumed, the intake of these elements was calculated. The food spilled was separated from the excreta, and deducted from intakes. In these experiments it was so small as to be practically negligible. Only a small portion was placed in the feeding cup at one time; the small amount remaining each day was thoroughly mixed with the new portion, to insure consumption of all constituents. The urine and feces were analyzed for calcium and phosphorus. At the age of 170 days in the first experiment and 133 days in the second experiment the animals were x-rayed, etherized, and killed by bleeding. Litter mates, fed the diet B of Sherman, served as controls.

Analytical methods. The blood serum was analyzed for calcium by the method of Clark and Collip (7), and for serum inorganic phosphorus by the method of Kuttner and Cohen (8). The femurs were dissected, dried, extracted with alcohol and ether, and the ash determined (3). The ash of the femurs was also analyzed for calcium and phosphorus. The remainder of the rat was incinerated and ashed. The ash was dissolved in HCl, and calcium and phosphorus determinations were made upon aliquots. The calcium was precipitated as the oxalate after adjustment to pH 5.0 to 5.6 and titrated with permanganate. The phosphorus was precipitated as the molybdate, treated with formaldehyde, and titrated with HNO_3 and

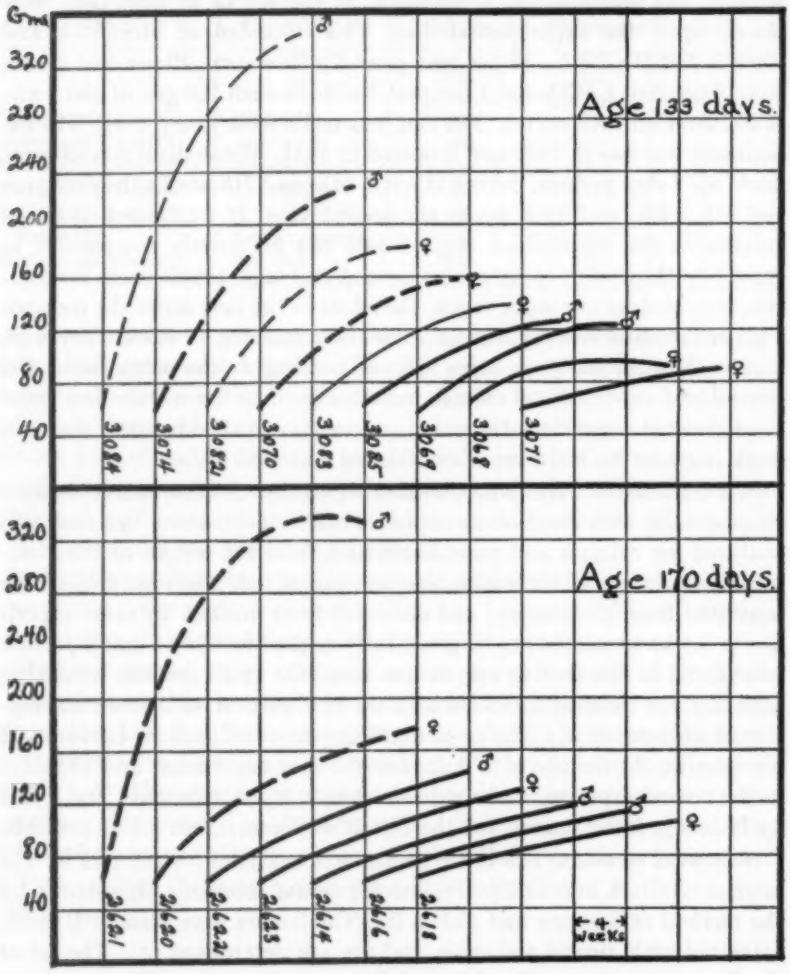

FIG. 1.—The solid lines represent growth curves of animals fed Steenbock and Black's diet No. 2965; the broken lines, the growth curves of those fed Sherman's diet B. In the upper section, the light lines refer to rats in stock cages and the heavy lines to those in metabolism cages throughout the experiment. In the lower section all curves represent the animals which were in metabolism cages for only the 5th, 6th and 7th weeks. All weight curves begin at 28 days of age.

NaOH. The accuracy of the methods of tissue and food analysis was of the order of 1 per cent.

Progress. The experiment proceeded with no known errors. All the animals appeared normal, except one that died of acute retention of urine and was discarded. The fur and eyes were in good condition. The activity of the experimental animals was similar to that of the controls. They appeared normal except that they were slenderer and smaller.

Results

The x-ray pictures showed that no rickets developed and, by this criterion, the bones were normal but smaller and thinner than those of the control animals.

The increase in weight of the experimental animals is charted in Fig. 1. This shows clearly that the experimental animals were definitely stunted compared to the normal controls. The weights of both males and females were below those of the control females. Whereas for both series the control males gained 12 to 13 gm. a week, the control females increased 6 to 7 gm. weekly and the experimental males and females averaged 4 and 2.5 gm., respectively, i.e., only 30 to 40 per cent of normal growth.

It is clear from Fig. 1 that at 133 days the animals in the metabolism cages did not gain as much as those in the stock cages. However, the same relations between diet and growth are evident whether under similar or dissimilar conditions. The changes which this alteration in weight brought about will be manifest when the analysis of the bodies is discussed.

We have previously shown that the experimental diet permits good growth when phosphate is added (9). Therefore the conclusion must be drawn that the phosphorus deficiency is the limiting factor in the growth.

The blood serum content of calcium of both normals and controls throughout the period investigated remained at the level of 10 mg. per cent. The inorganic phosphorus of the serum, as in humans, diminishes at maturity. In the first few months of life on the control diet values of 8 to 10 mg. per cent were found; at 130 to 135 days 6 to 7 mg. per cent and at 170 days 5 mg. per cent; the experimental animals at the same time showed 4.7 and 4.0 mg. per cent, respectively. Thus on the low phosphorus diet, even though cod liver oil was consumed, the phosphorus value was slightly lower than that found in the controls, but above the level found in rickets.

The bone ash. The ash percentage of the extracted femurs given in Table II shows 63.5 per cent at 133 days for the normals (average of males and females) and 56.2 per cent for the experimental animals; at 170 days 61.0

TABLE II
BONE ANALYSIS PER SINGLE FEMUR

Rat No.	Sex	Wet wt.	Dry wt.	Fat-free wt.	Ash of fat-free bone						
							Calcium		Phosphorus		Ca:P
		mg.	mg.	mg.	mg.	per cent	mg.	per cent	mg.	per cent	
Age 133 days. Sherman's diet B.											
3074*	♂	633	435	390	247	63.4	95	38.5	44	17.8	2.16
3084†	♂	804	546	499	319	63.9	118	37.2	55	17.2	2.15
3070*	♀	440	330	305	189	62.2	74	39.0	34	18.0	2.17
3082†	♀	522	377	342	217	63.6	82	37.8	39	18.0	2.11
Age 133 days. Steenbock and Black's diet No. 2965, plus cod liver oil.											
3069*	♂	387	258	222	116	52.6	45	38.6	20	17.3	2.25
3085*	♂	403	237	206	116	56.2	42	36.2	20	17.3	2.10
3068*	♀	342	225	196	107	54.9	43	40.0	19	17.8	2.26
3071*	♀	278	200	172	98	57.2	37	37.8	18	18.4	2.06
3083†	♀	416	288	255	154	60.5	59	38.2	27	17.5	2.18
Age 170 days. Sherman's diet B.											
2621‡	♂	1040	676	652	406	62.2	157	38.7	74	18.2	2.13
2620‡	♀	585	384	364	218	59.8	85	38.9	40	18.3	2.12
Age 170 days. Steenbock and Black's diet No. 2965, plus cod liver oil.											
2614‡	♂	419	255	233	131	56.3	49	37.5	24	18.3	2.04
2616‡	♂	420	256	224	118	52.8	46	39.0	21	17.8	2.19
2622‡	♂	500	316	288	161	55.8	65	40.3	29	18.0	2.19
2618‡	♀	420	271	245	143	58.4	54	37.8	25	17.5	2.16
2623‡	♀	480	313	298	162	54.4	64	39.4	29	17.9	2.20

* In metabolism cage for duration of experiment.
† In stock cage.
‡ In metabolism cage for 5th, 6th, and 7th weeks of life.

per cent for the controls and 55.5 per cent for the experimental animals. The values for the normals are in agreement with those found by other investigators but those of the experimental animals are too low to be classed as normal and too high to fall in the zone of values found in rachitic animals.

At 133 days the animals in the metabolism cages were smaller than

those in the stock cages and had lighter bones. The actual weights of the bones of the experimental animals, whether fresh, dry, or extracted, or of the ash, calcium, and phosphorus, were about 55 to 60 per cent of the normal. Closer analysis shows that, compared to the controls, the bones of the experimental rats were actually increased in fat, relatively richer in water and organic material and poorer in ash.

At 170 days the same general relations held except that the stunting effect of the rachitogenic diet was more marked and here the values of the bones reached only 30 to 40 per cent of that of the controls. Thus the growth of the femur closely resembles the body increase in weight. The bones of both the experimental and control groups comprise the same fraction of total weight.

A considerable amount of data has accumulated as to the composition of the ash of the bones in respect to the proportion of calcium to phosphorus. The whole question of the physical chemistry of bone formation and the possible mechanism involved has depended upon the composition of bone. We have heretofore merely determined the total ash, on the assumption that the composition with respect to calcium and phosphorus was constant within the limits of accuracy required. In this experiment, because of the long duration on low phosphorus diet, this hypothesis was subjected to a very severe test. It seemed desirable therefore to determine calcium and phosphorus separately. Analysis of the ash of the bones of the control animals, extracted with alcohol and ether, at 133 and 170 days of age gave approximate values of 38 per cent calcium and 18 per cent phosphorus with a Ca:P ratio of 2.14. The experimental animals, male and female, gave values which were the same, within the errors of the methods. Thus one must conclude that the mineral salts of bone have constant composition in respect to Ca:P even under the stringent conditions of this experiment. This is the second example of the law of the minimum. In this case only so much bone is formed as is permitted by the phosphorus content of the diet.

Metabolism studies. The intake of calcium and phosphorus of animals fed Sherman's diet B differs from that which we have previously reported normal with other diets (9). This diet has a Ca:P of 0.6 to 0.7 and is therefore a high phosphorus diet. We have previously discussed the significance of this factor in the diet and come to the conclusion that in a normal diet the Ca:P should be between 1 and 2, probably between 1.5 and 2 and that 2 was the best value available.

The data for Sherman's diet B for the 6th, 9th, 14th, and 18th weeks of

TABLE

METABOLISM OF CAL

Figures in terms of

Rat No.	Sex	Age	Calcium					
			Intake	Urine	Feces	Total output	Retention	
		weeks	mg.	mg.	mg.	mg.	mg.	per cent
								Sherman's
2632	♂	5–7	272	7	11	18	254	93
2634	♂	5–7	230	6	12	18	212	92
2637	♂	5–7	256	5	13	18	238	93
Av.	♂	5–7	253	6	12	18	235	93
2631	♀	5–7	233	7	8	15	218	94
2633	♀	5–7	212	6	7	13	199	94
2638	♀	5–7	226	8	15	23	203	90
Av.	♀	5–7	224	7	10	17	207	93
3074	♂	8–9	305	7	58	65	240	79
3070	♀	8–9	243	8	38	46	197	81
3074	♂	13–14	191	6	117	123	68	36
3070	♀	13–14	180	5	76	81	99	55
3074	♂	18–19	173	6	149	155	18	10
3070	♀	18–19	177	5	117	123	54	30
								Steenbock and Black's diet
Av.*	♂	4–5	586	90	390	480	106	18
Av.†	♀	4–5	570	72	412	484	86	15
Av.*	♂	5–6	662	102	272	374	288	43
Av.†	♀	5–6	759	51	267	318	441	58
Av.*	♂	6–7	597	84	292	376	221	37
Av.†	♀	6–7	684	106	339	445	239	35
3069	♂	8–9	492	159	193	352	140	28
3085	♂	8–9	468	112	223	335	133	28
3068	♀	8–9	684	197	301	498	186	27
3071	♀	8–9	480	107	172	279	201	42
3069	♂	13–14	397	120	173	293	104	26
3085	♂	13–14	508	143	255	398	110	22
3068	♀	13–14	397	102	167	269	128	32
3071	♀	13–14	422	104	205	309	113	27
3069	♂	18–19	437	140	299	439	−2	—
3085	♂	18–19	578	177	322	499	+78	13
3086	♀	18–19	519	205	261	466	+53	10
3071	♀	18–19	437	175	329	504	−67	—

* Rats no. 2614, 2616, 2622.

† Rats no. 2618, 2623.

III

CIUM AND PHOSPHORUS

one rat per week

Phosphorus						Retention Ca:P
Intake	Urine	Feces	Total output	Retention		
mg.	mg.	mg.	mg.	mg.	per cent	
Diet B.						
391	124	59	183	208	53	1.22
332	121	50	171	161	48	1.32
370	72	59	131	239	65	1.00
364	106	56	162	202	55	1.16
335	118	47	165	170	51	1.28
306	111	43	154	152	50	1.31
325	124	46	170	155	48	1.31
322	118	45	160	162	49	1.28
475	114	92	206	269	57	0.89
378	89	81	170	208	55	0.95
297	99	110	209	88	89	0.77
280	66	67	133	147	52	0.67
267	91	116	207	60	30	0.30
274	60	93	153	121	44	0.45
No. 2965, plus cod liver oil.‡						
123	5	83	88	35	28	3.03
120	5	80	85	35	24	2.46
139	5	79	84	55	40	5.24
160	5	80	85	75	47	5.88
126	5	78	83	43	34	5.14
144	5	83	88	56	39	4.26
121	4	65	69	52	43	2.70
115	4	64	68	47	41	2.83
168	4	81	85	83	49	2.24
118	4	51	55	53	45	3.80
75	9	54	63	12	16	8.70
96	9	65	74	22	23	5.00
75	9	47	56	19	25	6.70
80	9	54	63	17	21	6.60
85	4	88	92	−7	—	—
113	4	88	92	+21	19	3.70
101	4	86	90	+11	11	4.80
85	4	85	89	−4	—	—

‡ Rats were weaned at 21 days of age, and continued on Sherman's diet B until 28 days of age, when the experimental diet was begun.

life, given in Table III, show several interesting features. The food intake was maximal at the 9th week. Throughout these periods the calcium excretion in the urine was nearly constant; 5 to 8 mg. per rat per week, or 2 to 3 per cent of the intake. Differing from our previous experience with other normal diets (with a Ca:P = 2.0), the urinary calcium was in all cases less than the phosphorus.

The fecal calcium was also low throughout but especially in the period of the 6th week. Here the calcium in the feces represents less than 5 per cent of the intake. Again differing from our previous experience with other normal diets relatively higher in calcium, the fecal calcium was less than the fecal phosphorus for the first two periods. For the last two periods the calcium in the feces was greater than the phosphorus. As the fecal calcium increased with age, the fecal phosphorus also rose and smaller percentages of the calcium and phosphorus were found in the urine. The small amount of calcium in the urine and feces might be expected to result in high Ca:P retention. But such is not the case. Although during the 6th week 93 per cent of the calcium was retained, this dropped to 80 per cent in the 9th week, 36 to 55 per cent in the 14th week, and 10 to 30 per cent in the 19th week. The retention of the phosphorus remained at about 50 per cent throughout the period of observation. This resulted in diminishing ratios of Ca:P retained. Even for the first period the Ca:P ratios were only 1.16 and 1.28. We have considered 1.5 to 1.7 as normal at that age. The rest of the values, which are below 1.0, represent *high phosphorus* retentions.

Upon the experimental diet with a Ca:P of 4.0 to 5.3 the findings are in contrast to those for the controls and similar to our previous experience with high calcium low phosphorus diets. The urine was nearly phosphorus free and the fecal calcium was far greater than the fecal phosphorus in every case. The percentage of retention and the actual retention of both calcium and phosphorus were greater during the earlier periods of study than at the 14th and 19th weeks. In the last period negative balances of calcium and phosphorus were found in two cases.

The Ca:P retentions show consistently high ratios throughout, similar to those observed in the course of development of rickets, and represent, except in the last period where negative balances occurred, an *excess calcium* retention. In general the type of retention reflects the relation of minerals in the diet.

Analyses of whole animals. Whereas metabolism studies should indicate what is occurring during the period of study, the ash of the whole body should give information about the summation of all that has gone before.

Sherman and Booher (10) have recently used the ash method for biological assays of various types of diets, especially those of low and varying calcium content. Sherman and Quinn (11) have studied also the phosphorus content of male and female rats fed diet B for a long time. McCann and Barnett (12) have analyzed the bodies and bones of rats fed for four weeks a rickets-producing diet with and without vitamin D.

The values for the body content for calcium and phosphorus, shown in Table IV, were obtained by adding the amount found in the femurs and

TABLE IV

ANALYSIS OF TOTAL BODY FOR CALCIUM AND PHOSPHORUS

Rat No.	Sex	Diet§	Net body					
			Weight	Calcium		Phosphorus		Ca:P
			gm.	gm.	per cent	gm.	per cent	
Age 133 days.								
3074*	♂	Sherman	206	2.54	1.23	1.42	0.69	1.79
3069*	♂	Steenbock	108	1.30	1.20	0.74	0.69	1.76
3085*	♂	Steenbock	111	1.29	1.16	0.76	0.69	1.70
3070*	♀	Sherman	143	2.12	1.48	1.14	0.80	1.86
3068*	♀	Steenbock	83	1.29	1.56	0.73	0.88	1.77
3071*	♀	Steenbock	79	1.18	1.49	0.67	0.85	1.76
3084†	♂	Sherman	315	3.07	0.98	1.74	0.55	1.76
3082†	♀	Sherman	166	2.11	1.27	1.16	0.70	1.82
3083†	♀	Steenbock	128	1.63	1.27	0.92	0.72	1.77
Age 170 days.								
2621‡	♂	Sherman	294	3.40	1.16	1.99	0.68	1.71
2614‡	♂	Steenbock	104	1.38	1.32	0.76	0 73	1.82
2616‡	♂	Steenbock	105	1.34	1.28	0.74	0.71	1.82
2622‡	♂	Steenbock	132	1.66	1.26	0.94	0.71	1.77
2620‡	♀	Sherman	156	2.28	1.46	1.27	0.81	1.80
2618‡	♀	Steenbock	98	1.50	1.53	0.80	0.82	1.87
2623‡	♀	Steenbock	115	1.71	1.49	0.94	0.82	1.82

* In metabolism cage for duration of experiment.

† In stock cage.

‡ In metabolism cage for 5th, 6th, and 7th weeks of life.

§ The diets were Sherman's diet B and Steenbock and Black's diet No. 2965 plus cod liver oil.

that in the remainder of the body. They show the well-known sex differentiation—a greater per cent of minerals occurs in the females. The control rats in the stock cages were heavier not only than those in the metabolism cages but also than Sherman's animals (11) and show a slightly smaller per cent of both calcium and phosphorus. If these analyses were on a fat-free basis, closer agreement would probably result. The principal deduction to be drawn, in agreement with McCann and Barnett, is that *the ratio of total body calcium to phosphorus is essentially the same for males and females, experimental and control, on the high phosphorus and low phosphorus diets.*

This is an extension of the two previous examples of the law of the minimum. Only so much growth is permitted as allows the composition of similar (but not necessarily constant) structure. Minor differences which our methods do not reveal as significant differences, may be present. Another law may be operative. We know that tissues and body fluids may vary in certain minor but important respects (such as to justify the determinations we make daily in the laboratory for the diagnosis of disease). Such observations have been made in rickets recently by Haury (13) and Cole and Koch (14).

However, if one assumes that 99 per cent of the calcium is in the skeleton, the phosphorus associated with it can be computed. If one assumes a constant relation of Ca:P in all the bones, the total calcium in the body, divided by 2.14, equals the phosphorus in the bones. The remaining phosphorus is that distributed throughout the body. We have made such calculations and find no significant differences in the phosphorus of the soft parts of the rats fed the two diets. We have analyzed the tissues of rats on high calcium low phosphorus diets with and without vitamin D and have found no difference in phosphorus by the methods we used.

How then can the results of the metabolism and the analyses be compared? By the former methods the rats on the high calcium low phosphorus diets continue throughout the experimental period to have high calcium low phosphorus retentions. By the latter methods the composition of the body in respect to per cent of calcium and phosphorus and hence in their proportion to each other is normal. The results of the two methods are divergent. The explanation presents an unsolved problem.

The experimental animals are still laying down bone as rapidly as the limiting phosphorus in the diet permits, whereas the normal animals have reached complete development of their skeleton and have no longer a need for extra calcium. As far as bone deposition is concerned, the experimental animals are growing and the normal animals are adult.[1]

[1] Footnote p., 283.

SUMMARY

On a high calcium low phosphorus diet, in which the moderate content of vitamin A and deficiency in D is supplemented by vitamins A and D, growth is retarded. The metabolism continues for 19 weeks to show a high calcium low phosphorus retention.

The fat-free bones are normal in respect to the relative amounts of calcium and phosphorus which they contain.

The body shows no alteration from the normal in respect to per cent of calcium and phosphorus and their relative proportion.

The phosphorus deficiency of this diet causes delayed growth and is the limiting factor.

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[1] After the manuscript was sent to the editors, the article by Kletzien, Templin, Steenbock, and Thomas, entitled "Vitamin D and the Conservation of Calcium in the Adult. I," appeared in the *Journal of Biological Chemistry*, **97**, p. 265. In their experiments they also used Diet No. 2965, alone and with Vitamin D supplied by irradiation; pregnancy and lactation were used as additional stress instead of growth. They state further, "On this same ration, we have observed a distinct tendency to the production of bones of lower ash content with young growing rats even when they received an abundance of vitamin D." Our work thus confirms their finding, and adds evidence of a different type.

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THE SPECTROGRAPHIC ANALYSIS OF MILK ASHES

By

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IN RECENT years considerable attention has been focused upon the physiological and nutritional significances of the so-called biologically rare elements. It is only natural, therefore, that milk should be among the materials examined for the presence or absence of inorganic elements which are biologically rare or not among the major inorganic constituents of living matter. Accordingly, Wright and Papish (1) made spectrographic analyses of a number of cow's milks from the United States and Great Britain. As a result they concluded that milk contains the following elements in relatively large quantities: calcium, magnesium, phosphorus, potassium, and sodium; and the following elements in smaller concentrations or "traces": aluminum, boron, copper, iron, lithium, manganese, silicon, strontium, titanium, vanadium, zinc, and very likely rubidium.

More recently Zbinden (2), also using the spectrographic method, analyzed a number of cow's milk and human milk ashes from widely distributed European sources. From his results he concludes that both cow and human milk contain regularly and in approximately constant although small quantities the following elements: aluminum, chromium, copper, iron, manganese, lead, tin, titanium, vanadium, and zinc. To this list he adds silver as a probable constituent of human milk.

The results of these two investigations agree with respect to seven elements, viz.: aluminum, copper, iron, manganese, titanium, vanadium, and zinc, but disagree with respect to eight other elements in that Wright and Papish report boron, lithium, rubidium, silicon, and strontium, which are omitted by Zbinden (possibly because the most sensitive lines of some of these elements were not in the spectral region examined), whereas Zbinden reports chromium, lead, and tin which Wright and Papish omit. In view of this disagreement it seems warranted to publish a third set of data on the biologically rare elements contained in milk.

Material

The materials[1] used in this study consisted of 19 samples of cow's milk. Sixteen of these were samples delivered to the laboratory in standard commercial one quart bottles of dairies located in Baltimore, Philadelphia, Pittsburgh, New York City, and the District of Columbia. Of these 16 samples 8 were labeled pasteurized, 3 unpasteurized, and 5 certified. Presumably all were regular commercial stock products. The remaining 3 samples were milks of 3 different cows of a certified milk-producing herd and were obtained by milking directly into previously acid cleaned and distilled water rinsed glass-stoppered pyrex bottles under conditions prevailing in certified milk-producing dairies. A sample of the feed mixture fed to these certified milk-producing cows was also obtained and analyzed.

Methods of Analysis

The methods of analysis were spectrographic and therefore fundamentally the same as those used by Wright and Papish and by Zbinden. Approximately ten gram portions of the materials, viz., the 19 milks and the feed mixture, were ashed at low temperature in both silica and platinum dishes directly over Bunsen burners in a relatively small room used only for that purpose at the time and therefore free from other fumes. During the ashing the room was furthermore kept as free as possible from air currents. Contamination of the samples seemed less likely by this procedure than by ashing in a muffle furnace. Immediately after ashing, the dishes containing the ashes were placed underneath glass cover dishes and stored in this condition until subjected to spectrographic analysis.

The technic employed in the spectrographic analysis of these ashes was essentially that described by Tourtellotte and Rask (3) except that graphite electrodes were used in addition to copper electrodes, and examinations were made of the spectra extending from 2400 to 6800 Ångström Units. The spectrograph used was the Hilger E-1, an instrument of particularly good resolving power throughout the ultraviolet and short visible regions. A total of at least three spectrograms was prepared of each ash. These spectrograms included, in the case of every ash, arc spectrograms with graphite and spark spectrograms with copper electrodes. These spectrograms were searched for "*raies ultimes*" of the elements listed in the following summary, which omits elements such as chlorine, iodine, etc. which are not detectable by ordinary spectrographic methods:

[1] Kindly supplied by Dr. J. H. Shrader, Director, Research Laboratories of the National Dairy Products Corporation.

Summary

1. The following elements were found in large quantities in all milk samples: calcium, magnesium, phosphorus, potassium, and sodium.

2. The following elements were found in smaller quantities (traces) in all milk samples: barium, boron, copper, iron, lithium, rubidium, strontium, titanium, and zinc. Of these barium is the only one which has not been reported previously.

3. The presence of aluminum and manganese was not definitely demonstrated, although these elements were present in the feed mixture.

4. The presence or absence of silicon and vanadium could not be established definitely owing to the presence of these elements in the graphite electrodes used.

5. The presence of antimony, arsenic, bismuth, caesium, cobalt, cadmium, fluorine (as the CaF_2 band), germanium, gold, indium, lanthanum, mercury, molybdenum, nickel, osmium, palladium, silver, zirconium, chromium, lead, and tin could not be detected, although Zbinden concludes that the last three of these elements, viz., chromium, lead, and tin, are present in European milks.

The following table shows how these results compare with those of Wright and Papish and those of Zbinden, omitting calcium, magnesium,

Mineral Elements in Milk as Detected by the Indicated Investigators

	Wright and Papish	Zbinden	Blumberg and Rask
Aluminum	+	+	?
Barium	−	*	+
Boron	+	*	+
Chromium	−	+	−
Copper	+	+	+
Iron	+	+	+
Lead	−	+	−
Lithium	+	*	+
Manganese	+	+	?
Rubidium	+	*	+
Silicon	+	−	?
Strontium	+	*	+
Tin	−	+	−
Titanium	+	+	+
Vanadium	+	+	?
Zinc	+	+	+

\+ Present
− Absent
? Questionable
* Not tested for

phosphorus, potassium, and sodium, concerning whose presence in milk there is no question.

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STUDIES ON THE ROLE OF ZINC IN NUTRITION

By

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THE simplest chemical composition of the complete diet of man and the higher animals is a subject of great theoretical as well as practical interest. The total number of pure chemical substances needed is not, as yet, known with certainty. Studies on nutrition since the earlier years of this century have disclosed the subtle influence of almost unbelievably small amounts of materials in the diet on the health of the animal and even the maintenance of life itself. The discovery and study of vitamins has done much to promote research toward the goal of successfully rearing animals on a diet the chemical composition of which is definitely known.

The field of study of the inorganic constituents of foods has been overshadowed by the interest in the study of vitamins; however, recent studies on the effect of the deprivation of certain elements have led to interesting results. Thus, copper (1) and manganese (2) have been found to be essentials for the complete diet, while fluorine (3), aluminum (4), and nickel (5) have been found to be dispensable.

Zinc, though one of the less usual elements, is almost universally present in plant and animal substances. Investigators who have examined carefully enough for its presence have reported zinc in a great variety of biological materials. "Man ingests in his food practically as much zinc as he does iron." (6) This would tend to indicate that zinc has some nutritional function. It is, therefore, an interesting question as to what would happen to an animal deprived of zinc during its lifetime.

This question has been studied before but without conclusive results. Bertrand and Benson (7) conducted feeding experiments on mice with a zinc-free diet. This diet was composed of starch, casein, cellulose, lactose, cocoa butter, and a salt mixture. It lacked all the vitamins and, naturally, the animals failed. Animals fed on this diet lasted about 15 to 20 days,

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while others fed the same ration with the addition of one hundredth of a gram of zinc per hundred grams of food lived 25 to 50 per cent longer.

McHargue (8) tried to find out whether growth would take place on diets deficient in copper, manganese, and zinc. There was a complete lack of vitamins. He did not attempt to remove the zinc from the diet but added the metal to a basal ration low in zinc. He found some favorable effects on the time of survival due to the addition of 25 parts per million of zinc.

Both the above studies are so complicated by a lack of the essential dietary principles that they cannot be in any way accepted as proving a favorable action of zinc. Thus it was that Hubbell and Mendel (9) attempted again to solve the question of the importance of zinc in a complete diet. They found, however, that it was practically impossible to free their casein and their source of water-soluble vitamins from it. These contained, as used, 4 and 14 parts of zinc per million respectively. Therefore they had to content themselves with feeding a zinc-low diet furnishing mice with about 0.005 mg. per day. Assuming mice to eat about 3 gm. of food daily, this would be a concentration of about 1.67 parts per million of zinc. They fed groups of controls the same ration with the addition of 0.02 and 0.04 mg. of zinc per day and also a control diet which was estimated to furnish 0.318 mg. of zinc per day. Great care was taken to exclude dust from the cages for that was found to be a source of zinc. As a result of this experiment they found that there was a slight retardation of growth when the animals were fed on a zinc-free diet and the addition of zinc caused a slight stimulation to growth, but it was not sufficient to make the diet equal to standard. No considerable differences were observed. The animals fed on the zinc-low diet showed a distinctly lower zinc content than those on the control diets. A decrease of from 0.021 to 0.016 mg. per gram for the females and from 0.019 to 0.012 for the males was noted. Those animals which received slight additions of zinc showed some increase in body zinc but the controls receiving 0.318 mg. per day had a smaller zinc content per gram of mouse than those receiving 0.224, 0.04 or 0.02 mg. per day. There did not seem to be any storage of zinc in the actively growing mouse. Hubbell and Mendel conclude that it is possible that the value of zinc lies not alone in the metal itself but it may be in some way associated in function with other metals present in small amounts. It is not unlikely, however, that there is some variation in growth with varying amounts of zinc and the metal is not merely an accidental factor in the nutrition of the mouse.

Thus it is that, in spite of the widespread occurrence of zinc in plant and animal organisms (10), no one has demonstrated conclusively any specific effect of this metal in animal nutrition. For this reason and as a part of an extended series of studies on mineral metabolism, we have studied the effect of practically zinc-free diets on the growth and health of rats.

Experimental Work

The preliminary studies of foodstuffs were made by means of spectrography. The apparatus and main details of the method of its use have been described by Tourtellotte and Rask (11). The sample of material to be examined was charred in a low, wide silica dish on a hot plate, ignited in a muffle at 450°C. as measured by a pyrometer. This procedure was shown to be satisfactory by Thompson (12) and also recommended to the Association of Official Agricultural Chemists (13). When the sample did not yield a white ash after 4 or 5 hours, it was treated with a few cubic centimeters of redistilled nitric acid, evaporated to dryness and the heating continued. The extraction of the ash through filter paper was avoided. The white ash was washed into a small beaker and the silica dish rinsed with a few cubic centimeters of concentrated redistilled sulfuric acid which was added to the contents of the beaker. A little redistilled nitric acid was added to oxidize the iron and the solution evaporated to the appearance of white fumes. It was then allowed to cool, diluted to about 75 cc. with distilled water, and approximately tenth normal zinc-free sodium hydroxide was added until the solution was alkaline to phenolphthalein with one or two cubic centimeters in excess. This solution was electrolyzed in the cold with constant stirring using a platinum anode and two pure copper rods as the cathode. The rods were dipped about one centimeter into the solution and a current of about five hundredths of an ampere was passed through them for 3 hours. They were then rinsed with distilled water and dried in the air. They were stored in test tubes until ready for use in spectrography. The rods were used as electrodes for the arc or spark and the light from this source was photographed by a Hilger E-1 spectrograph.

Little difference was detected in the sensitivity of the detection of zinc whether the arc or spark was used to excite the spectrum. The spark was preferred since the lines did not fog over on the adjacently photographed spectrum.

The photographic plates were examined with a hand lens having a power of fourteen diameters. The lines examined for were 4811 Å, 4722 Å and 4680 Å. Twyman (14), in his "Wavelength Tables" reproduces de Gra-

mont's tables of the *raies ultimes* (lines of greatest sensitivity) in which the first two lines are given as the *raies ultimes* of zinc. In all work the spectrograph was set so that these lines were at minimum deviation.

The sensitivity of the method was tested by electroplating zinc from solutions of known strength and examining the spectra from these plates. The effect of ashing was ascertained by adding known amounts from 0.0001 mg. to 1.0 mg. of zinc to ten gram samples of sugar and then carrying out the procedure as described above. Amounts of zinc down to 0.001 mg. could be detected. This is not as sensitive as the results of Bayle and Amy (15) who detected 10^{-8} gm. of zinc by a similar method.

Only the crudest quantitative estimations were attempted by this spectrographic method. Its main purpose was to furnish definite assurance that the substance observed was actually zinc. The line 4811 Å was found present faintly and by itself in the spectrograms from 0.001 mg. of zinc. In a ten gram sample, as usually taken, this would represent a concentration of 10^{-7} of zinc. When the spectra of the ash of the diets showed the absence or only faint presence of this line they were called zinc-free.

In the instances where quantitative determinations were desired the turbidimetric method of Fairhall and Richardson (16) was employed. However, this method gave trouble in the cases where the whole bodies of large rats were analyzed. The calcium was precipitated as citrate in the first adjustment of the acidity and this carried through the subsequent precipitations of zinc to the final turbid solution where it produced an interference. In solutions having less calcium the method yielded good results.

Both the spectrograph and the turbidimetric method agreed that the concentration of zinc in the zinc-free diets was much less than 1 part per million.

The spectrograph showed traces of zinc to be present in the following food substances taken from the stock at hand: casein, dextrin, butter, wheat germ oil, raw starch, starch washed twice with two per cent hydrochloric acid, starch washed four times with 5 per cent hydrochloric acid, viosterol, and both 50 and 70 per cent alcoholic extracts of yeast. No zinc was found at any time in the salt mixtures. Thus it was found necessary to purify practically all the foods fed in this experiment.

An attempt was made to have the diet as simple as possible. In brief, the essentials were thought to be a suitable protein, carbohydrate, linoleic acid, salts, vitamins A, D and E, and the water-soluble vitamin B and G complex.

Protein

Skim milk was electrodialyzed in a porous clay battery jar eleven inches high and four inches in diameter. A platinum anode surrounded by an alundum tube eleven inches long and one inch in outside diameter which was supported by corks on a long glass stirring rod was placed in the center of the above jar and constantly rotated by a motor. The jar was wrapped with copper fly screening to serve as a cathode and was set in a copper can through which a stream of tap water was passed during the electrodialysis. This apparatus would hold about a liter and a half of skim milk. A current of about 1.4 amperes was passed through the milk and after about two hours the casein would float as a coagulated mass leaving a yellowish liquid. The liquid was then strained off through a piece of muslin, the casein collected and stored in alcohol until sufficient amount was obtained. Since alcohol may contain zinc it is well to redistill all of that used for this purpose. Contamination of one batch of casein was thought due to the lack of this precaution. The casein was dried by spreading in flat pyrex dishes and passing over it a current of warm air. By making three or four runs a day about a gallon and a half of milk could be used and 200 grams of air dry casein produced.

The casein so prepared was spectrographically free from zinc.

Carbohydrate

Zinc seemed to be intimately bound in the starch granules so that it was impossible to obtain zinc-free starch. For this reason sucrose was employed as the source of carbohydrate. Its effect in the diet had been investigated by Jackson (17) who reported little, if any, harmful effects in proportions as high as 80 per cent. Commercial sugar was found to have a low but variable zinc content. Merck's C.P. sucrose was found to have a zinc content of the order of 10^{-7} part. This was used to make up the zinc-free diet.

Salts

The stock salts 51 of this laboratory were found to be free from zinc when tested either as separate salts or as a mixture. Their composition is as follows:

	parts per hundred of food
Calcium carbonate	1.5
Potassium chloride	1.0
Sodium chloride	0.5

Sodium bicarbonate	0.7
Magnesium oxide	0.2
Ferric citrate	0.5
Monopotassium phosphate	1.7
	6.1

Manganese occurred as an impurity in the above mixture.
Copper was added to the diets separately as described later.
Iodine solution was added to the drinking water once every week.

Fats and Vitamins A and E

Butter was chosen to provide these essentials. Simmonds, Becker, and McCollum (18) found diets containing 5 per cent of this to promote fair to excellent nutrition and fairly good reproduction. Similar diets containing 10 per cent of butter fat resulted in good nutrition and normal deliveries.

Butter fat contains appreciable amounts of zinc. This was removed by dissolving the butter fat in dichlorethylene, washing four times with approximately normal hydrochloric acid, twice with water and drying over sodium sulfate. The dichlorethylene was then distilled off in vacuo at about 55°C. Later the use of the solvent was discontinued for the butter could never be freed from a smell of it and it was thought to have some effect on the growth of the animals. The butter fat was washed while in a melted state instead of being dissolved. The first lot of butter lost its vitamin potency in 4 months even though kept in a refrigerator, so in later experiments only enough butter for 3 or 4 weeks' use was made up at a time.

Vitamin D

The ordinary viosterol was found to contain zinc; however, this was all removed by four washings with normal hydrochloric acid in the manner described for the treatment of butter.

The Water-soluble Vitamins

This group presented the greatest difficulty, and failure to make a satisfactory preparation of them has probably been at the bottom of the nutritional insufficiencies that occurred in the feeding experiments.

Attempts were made to free yeast extracts of zinc by electrolysis and by precipitation with hydrogen sulfide in the presence of copper acetate, but without success. An attempt to raise bakers' yeast in a synthetic medium lacking in zinc produced only a small growth of yeast which had no vitamin

potency. The lack of vitamin potency was not connected with the absence of zinc from the medium, for the addition of zinc to the same medium caused an increase in growth of yeast, but this yeast had no beneficial effect when fed to rats suffering from a vitamin B deficiency.

The source of vitamins B and G finally adopted was a modification of the preparation of Seidell (19). Dried yeast[1] was suspended in 50 per cent alcohol in the ratio of one liter of alcohol per 100 gm. of yeast. Alcohol was used since water extracts were found to be impossible to filter. This suspension was put into three-liter flasks and hydrogen sulfide run in until the suspension seemed saturated. The flasks were shaken frequently. They were then stoppered tightly and allowed to stand over night. The liquid was filtered off through coarse paper and treated with 37.5 gm. of Lloyd's reagent for each hundred grams of yeast taken. The suspension was stirred mechanically for one hour and then the liquid was filtered off through ashless paper and discarded. The activated Lloyd's reagent was sucked as dry as possible and, in later experiments, dried in a current of warm air, ground and fed in the diet. During the first feeding experiments the vitamins were still further extracted from the activated reagent by ice-cold 2 per cent sodium hydroxide which was quickly neutralized by hydrochloric acid. The resulting solution was evaporated on a boiling water bath in vacuo and the concentrated liquid poured over a measured amount of casein and dried in a current of warm air. This process added a considerable amount of sodium chloride to the diet, lowered the potency of the vitamins and was tedious. It was found later that the activated reagent was practically zinc-free in itself, so further extraction was discontinued. Copper sulfate solution calculated to produce ten parts per million of copper in the diet was added to the vitamin preparation before it was dried. Zinc dissolved in hydrochloric acid was added in the same manner when so desired.

The Animals and their Care

The animals were obtained from a mixed colony of rats (*Mus norvegicus*). There were four strains,—white, yellow, brown, and black, which had been interbred for 19 years so that they are quite uniform in rate of growth and reproduction. They were started on the experimental diets a short time after being weaned, when their weights were between 35 and 50 gm. Five of them were kept in a cage and no bedding was supplied except when females had young. The female was put in a separate cage and strips of filter paper were then supplied for building a nest.

[1] Northwestern Yeast Co.

The cages for the zinc-free rats were made from iron hardware cloth of three meshes to the inch. This had not been galvanized and was spectrographically free from zinc. Large tinned cans, also zinc-free, were cut up and used for the frame work. The feed cups were tin plated sheet iron and were spectrographically zinc free.

All the cages had wire netting false bottoms to prevent the animals from eating their feces and thus taking in again any zinc excreted by that route. The wire bottoms had the further advantage of minimizing any contamination due to dust. For this same reason all the zinc-free cages had solid tops which prevented dust from settling in them. The control rats receiving zinc were kept in galvanized cages of approximately the same size as the zinc-free ones. These too had wire false bottoms.

The animals were supplied with food and water in abundance. The solid food materials were ground to a fine powder and thoroughly mixed. The butter fat was melted in warm water, the viosterol added to it, and incorporated into the mixture. The animals therefore could not select any one component of the diet.

The water used was the ordinary laboratory distilled water from a block tin still. No zinc was found in a hundred cubic centimeters of it.

Diets

Diet I.

Electrodialyzed casein	180 grams
Yeast grown on a zinc-low medium	50
Acid-washed butter fat	80
Salts 51	61
Sucrose, Merck's C.P.	629
	1000
Viosterol	15 drops

Diet II was a control on Diet I and was of the same composition with the addition of 20 mg. of zinc in the form of its sulfate.

Diet V.

Electrodialyzed casein	180 grams
Acid-washed butter fat	50
Salts 51	61
Sucrose, Merck's C.P.	609
	900
Yeast extract equivalent to 100 gm. dried yeast	
Viosterol	15 drops

Diet VI was a control on Diet V and was of the same composition with the addition of 200 mg. of zinc carbonate which contains roughly 100 mg. of zinc.

Diet VII.

Electrodialyzed casein	180 grams
Acid-washed butter fat	50–80
Salts 51	61
Sucrose, Merck's C.P.	609–579
	900

Yeast extract equivalent to 100 gm. dried yeast.

Viosterol	15 drops
Copper as $CuSO_4$	10 mg.

Diet VIII was a control on Diet VI and was of the same composition with the addition of 100 mg. of zinc.

Diet IX was another control on Diet VII and was the same with the addition of 20 mg. of zinc.

Diet XI.

Electrodialyzed casein	180 grams
Acid-washed butter fat	80
Salts 51	61
Sucrose, Merck's C.P.	579
	900

Activated Lloyd's reagent equivalent to 150 gm. of dried yeast

Viosterol	15 drops
Copper as $CuSO_4$	10 mg.

Diet XII was a control on Diet XI and was of the same composition with the addition of 20 mg. of zinc. Later stock acid-washed casein was substituted for the electrodialyzed casein.

Diet XIII was the same as Diet XI with the addition of 100 mg. of zinc. Later stock casein was substituted as in Diet XII.

Diet XIV replaced Diet XI and was of the same composition except that stock acid-washed casein was used.

Diets I, V, and VII were found by the spectrograph to have no zinc or at times slight traces corresponding to amounts of the order of 10^{-7} part. The casein used in preparing Diet XIV and later samples of Diets XII and XIII was found by analysis by the turbidimetric method to have a zinc content of 4.8 parts per million of zinc. Samples of Diet XIV showed a zinc content of 0.6 and 0.8 part per million.

Feeding Experiments

In the first instance 5 rats were fed Diet I and 5 others of the same age were put as controls on the zinc-containing Diet II. The growth failed with all the symptoms of vitamin B deficiency in both groups. Three of the zinc-fed and one of the zinc-deficient animals died of these symptoms. The surviving zinc-deficient animals were killed for analysis while the controls were used to assay other vitamin preparations. No significant effect on the symptoms of vitamin deficiency was observed due to the presence or absence of zinc.

In the second experiment the source of water-soluble vitamins was improved by using the yeast concentrate described before. Diet V was fed and Diet VI containing roughly 100 parts per million of zinc was used as a control. Four animals of the same age were put on each diet. However, in this experiment the butter fat used had stood in the ice box from January to April. It still had a smell of dichlorethylene and had turned white. The animals fed on this developed a severe xerophthalmia and, though they had not begun to decline in weight, they were not considered worth carrying over the summer and so were killed for analysis.

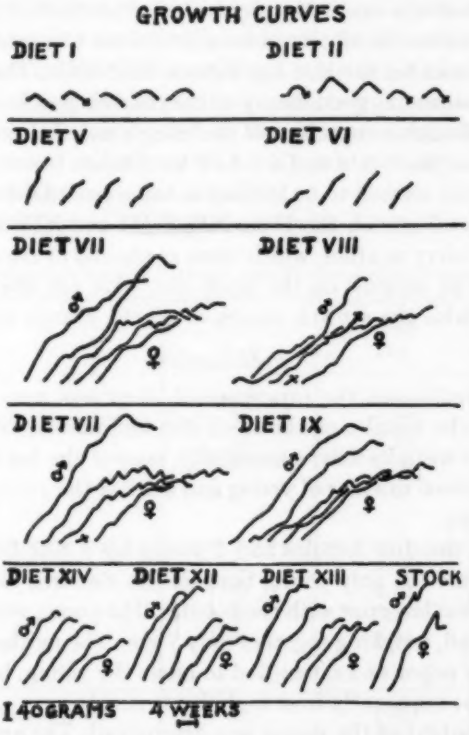
The butter fat had been cut to 5 per cent in this experiment, for the young rats on the previous diet had developed a very greasy condition of the fur. The amount of zinc in the control was raised to intensify any action which it might have on the growth of the rats.

No difference was observed in the rate of onset or the appearance of the symptoms of the xerophthalmia, so far as this condition had progressed before the animals were killed, between the zinc-deficient and the control zinc-fed animals.

A third set of animals was started on Diet VII with Diet VIII containing roughly a hundred parts per million serving as a control. At the start of the feeding period the weather was sultry and the rats obviously suffered. Thus the early growth curve is poor. After 12 weeks the butter fat was raised from 5 to 8 per cent and the sucrose cut down a corresponding amount. This had no noticeable effect on the growth but it was hoped that the added butter would supply more of the vitamin E.

Four weeks after the third group had been started on Diet VII the weather had moderated, so a fourth set was started on the same diet. However, the former control animals receiving Diet VIII, containing a hundred parts per million of zinc, were not growing well. It was thought possible that this amount was too much, so another control diet, Diet IX, was used to control this experiment. This diet contained roughly 20 parts per million of zinc.

The growth of the rats in this experiment was fairly satisfactory though it was not equal to the average for the stock rats in this colony. The almost complete absence of zinc (a concentration of the order of 10^{-7} or less) had, if any, a slightly beneficial effect as compared with the controls. The number of rats was too small, however, to make any statistical study. Nine rats were raised to the peak of their growth curve on the zinc-free Diet VII. Five controls fed 20 parts per million of zinc in the same diet grew slightly



less well, and 4 controls fed 100 parts per million of zinc made the poorest growth.

The limiting factor in the previous diets was believed to be the water-soluble vitamins. Diet XI was planned to correct this by raising the amount of activated Lloyd's reagent in the diet to the equivalent of 15 per cent of dried yeast. Groups of 5 young rats were started on this diet, and Diets XII and XIII, which contained 20 and 100 parts per million of zinc re-

spectively. At the end of the first ten days it was found that the new batch of electrodialyzed casein was contaminated with zinc. It was not possible to prepare more, so the stock acid-washed casein (washed 6 times with tap water and 7 times with 0.2 per cent acetic acid) was used to make up Diet XIV. This diet contained in the order of 1 part per million of zinc. Another group of 5 young rats was started on this diet while the rats which had been on Diet XI were transferred to a stock diet to serve as a control for the rate of growth of rats under the physical environment of the experiment.

This experiment was continued for a little over 13 weeks for the control rats and 12 weeks for the zinc-low rats on Diet XIV. The growth of this latter group was not as good as any of the controls particularly in the case of the males though comparison of the groups may be somewhat vitiated by the fact that those rats on Diet XIV were taken from a different litter.

The basal diet seemed to be lacking in some essential to optimal health for practically all rats on the Diets XII, XIII and XIV were affected by the period of sultry weather, which came at the end of the experiment, and lost weight. The animals on the stock diet were not affected to such an extent. Otherwise the growth curves of all the groups are quite similar.

Reproduction

The reproduction on the experimental diets was very unsatisfactory; while each of the females on the stock diet had normal deliveries and one was allowed to wean its litter successfully, none of the females on the other diets had a normal number of young and none of the young survived more than a few days.

One of the zinc-free females had 2 young by a zinc-free male on Diet VII. These were the only young born on the zinc-free diets even though a stock male was later put with the females. The young were born alive but were very small, weighing together only 7 gm. The mother was solicitous, built a nest of paper and attempted to nurse the young, but they died the following night apparently from inability to suckle.

The zinc content of the young was determined. The amount was about one-quarter of that found in normal stock young.

No young were born to rats on the diets containing 20 parts per million of zinc while 3 females on diets containing 100 parts per million of zinc gave birth to a total of 8 young. None of these young survived more than a day.

At the termination of the feeding period for the rats on Diets XII, XIII and XIV the animals were autopsied. One of the females on the zinc-low Diet XIV had 4 small fetuses in the uterus. Among the group on Diet XII

containing 20 parts per million of zinc, one female had 4 and another 2 fetuses. Among those getting Diet XIII containing 100 parts per million of zinc one female had 10 small fetuses while another had 9. This would tend to indicate that fertilization of the egg takes place on these diets and the subsequent failure to reproduce is due to the death of the fetus in utero.

Effect of the Zinc-free Diet on the Zinc Content of the Body

At the start of the first feeding experiment 4 rats of the same age as the experimental animals were killed for analysis. Their bodies were slit open and the whole rat dried without washing out the intestines. When the rats on Diet I were almost at the point of death from vitamin deficiency the surviving 4 were killed and dried in the same manner. Thus we have a comparison between animals raised on a stock diet and similar animals carried 6 weeks further on a zinc-low diet.

On the termination of the experiment with Diets V and VI the rats

ANALYSES OF RATS BY FAIRHALL AND RICHARDSON'S METHOD

	Weight of rat	Total zinc	P.P.M. zinc
	grams	mg.	
Stock rats at beginning of experiment..	59	1.20	20.0
	62	1.10	17.7
	62	1.00	16.1
	65	1.20	18.5
			av. 18.1
Rats on Diet I (zinc free).....	37	0.20	5.4
	49	0.40	8.2
	35	0.25	7.1
	66	0.50	7.2
			av. 7.0
Rats on Diet VI (100 p.p.m. zinc).....	79	1.60	20.3
	105	1.60	15.2
	120	1.90	15.8
	92	0.70	7.6
			av. 14.7
Rats on Diet V (zinc free).....	151	0.50	3.3
	109	0.45	4.1
	106	0.40	3.8
	94	0.70	7.4
			av. 4.7

ANALYSES OF YOUNG

Diet of parents	No. of young	Total weight	Total zinc	P.P.M. zinc
		grams	mg.	
VII	2	7	0.04	5.7
VIII	4	19	0.38	20.0
Stock	3	18	0.37	20.5

were killed and the lower intestines washed out with distilled water before the bodies were dried for analysis. Here we have a comparison of rats fed a zinc-free diet compared with mates of the same age fed the same diet with the addition of 100 parts per million of zinc over a period of 5 weeks.

The zinc content of the zinc-fed controls and the stock rats was roughly the same. Animals on the zinc-free diet lost not only in the proportion but also in the actual amount of zinc present in the body.

CONCLUSIONS

A diet has been prepared which supports growth in the rat and having a zinc content which is of the order of 10^{-7}, or less, part.

Zinc is probably not an essential nutritional factor in the growth of the rat.

Normal reproduction was not obtained on the experimental diets or controls but conception and successful gestation took place when both parents had been raised on a zinc-free diet.

The young born to a female on such a diet had a greatly diminished zinc content as compared with young born to a female on a stock diet.

The zinc content of the rat's body is somewhat mobile and depends, to some extent, on the content in the diet.

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THE CALCIUM RETENTION ON A DIET CONTAINING LEAF LETTUCE

By

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SINCE calcium is one of the minerals which is given first consideration in planning an adequate diet, it seemed of interest to investigate the calcium utilization of one of the commonly used fresh thin green leafy vegetables, namely, leaf lettuce (*Lactuca sativa*).

Human adults have served as subjects in the studies on the calcium utilization of various forms of milk (1 to 8) and also in the studies on the calcium utilization of plant foods (1, 2, 3, 9, 10). In the study of the availability of the calcium of spinach, McLaughlin (3) found it to be a satisfactory source of calcium for seven adult women when spinach furnished 70 per cent of the total calcium intake. The average calcium intake for these subjects was 9.1 milligrams per kilogram of body weight. Six subjects were in positive calcium balance and one was in equilibrium. Rose (1) reported, in a calcium retention study on carrots with four adult women on an average intake of approximately 5.9 milligrams of calcium per kilogram of body weight, positive calcium balances in all subjects except one, who had found the carrot diet slightly less satisfactory for calcium retention on account of digestive disturbances. One subject had nearly the same calcium retention when approximately 55 per cent of the total calcium intake was furnished by the carrots as when milk furnished 70 per cent of the total calcium intake. The results of a study on five healthy women, reported by Pittman (9), in which navy beans furnished 80 to 85 per cent of the total calcium intake, seemed to indicate that 5.5 to 6.5 milligrams of calcium per kilogram of body weight were insufficient for calcium equilibrium. When almonds, as reported by Rose and MacLeod (2), furnished 73 to 86 per cent of the total calcium intake for nine adult women, the subjects, with two exceptions, were practically in calcium equilibrium. The daily calcium intake per kilogram of body weight ranged from 4.1 to 9.4 milligrams of calcium. Blatherwick and Long (10) in a study including a mixture of vegetables, some of which were consumed uncooked, others cooked, and still others canned, found positive calcium balances when the calcium

intake of the two young women was approximately 9 and 13 milligrams per kilogram of body weight (calculated from data published). In their study this statement was made, "The commonly accepted values, as given in the different books by Sherman, were used in computing the calcium and phosphorus contents of the diets."

On a study of two adult dogs McClugage and Mendel (11) showed that the calcium of the vegetables, spinach and carrots, is not so well utilized as the calcium of milk. The calcium utilization of the dried spinach was very poor even on the intake as high as 30 milligrams of calcium per kilogram of body weight for dog B (calculated from data published). The same was true for Dog A when given 20 milligrams of calcium per kilogram of body weight per day. In the experiment in which dried carrots were used the calcium utilization was also poor for dog B when the calcium intake was 22 milligrams per kilogram of body weight per day. Dog A showed poor utilization when 15 milligrams of calcium per kilogram of body weight per day were furnished by the dried carrots. The investigators believed this might be due to the large amount of bulk in the diet, since the fecal mass increased 90 to 100 per cent when the vegetables were fed.

Sherman and Hawley (12) found the calcium balances of children, ranging from 5 to 13 years of age, less satisfactory when carrots and spinach replaced one-half of the milk as the source of the calcium in one series; and the same when carrots, spinach, and celery replaced one-half of the milk in another series.

Experimental Procedure

Two healthy young women, one an assistant in Nutrition in the School of Home Economics and the other a graduate student in Nutrition, served as the subjects. They were comparatively similar in weight, namely, 52.3 and 50.2 kilograms. They were well within the correct range of weight for their height, had lived on an adequate diet previous to these studies, and lived healthy, active lives. During these studies the subjects were engaged in the same activities.

Before participating in the lettuce study both subjects ate a quantity of lettuce which would be necessary to meet the calculated requirement for calcium equilibrium and which would represent a high percentage of the total calcium. During this time the subjects abstained from other vegetables and fruits. It was found that the subjects experienced no difficulty in consuming the large amount of this leafy vegetable for three consecutive days during February, nor did they experience any digestive disturbance.

The studies for the utilization of the calcium extended over a period of 18 days during March and April. The 18 days were divided into two periods of 9 days each, one for the study of leaf lettuce and the other for the study of pasteurized milk. Each 9-day period consisted of a 3-day preliminary period followed by a 6-day experimental period. During the preliminary period the same weighed amounts and kinds of food were eaten as those of the experimental period.

The diets were planned to furnish an amount of calcium that would be close to the calculated average requirement for equilibrium (13) and to meet the protein and energy requirements of the subjects. In Table I is given the amount of food consumed daily and also the calcium content of each food according to chemical analysis. The two subjects ate similar amounts of food. During the leaf lettuce experimental period, Subjects I and II consumed 54 grams protein, 83 grams fat, and 263 grams carbohydrate which yielded about 2,000 calories as calculated from Rose's Tables (14). During the pasteurized milk period Subject I consumed 53 grams of protein, 81 grams of fat, and 269 grams of carbohydrate which yielded about 2,000 calories. Subject II ate a similar calculated amount, namely,

TABLE I
DAILY FOOD INTAKE

Food	Leaf Lettuce Experimental Period				Pasteurized Milk Experimental Period			
	Subject I (52.3 kg.)		Subject II (50.2 kg.)		Subject I (52.3 kg.)		Subject II (50.2 kg.)	
	Wt.	Calcium	Wt.	Calcium	Wt.	Calcium	Wt.	Calcium
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Lettuce, leaf, E.P.	624	.493	595	.482	—	—	—	—
Milk, whole pasteurized	—	—	—	—	373	.451	369	.446
Apples, fresh, cored but unpared	—	—	—	—	400	.024	400	.024
Beef, lean round	185	.011	185	.011	150	.009	150	.009
Crackers, soda	75	.026	75	.026	75	.026	75	.026
Butter-fat	60	—	60	—	45	—	48	—
Sodium chloride C.P.*	2		2		2		2	
Sucrose	190	—	190	—	139	—	130	—
Total		.530		.519		.510		.505

* Calcium content of the sodium chloride, c.p., was determined and found to be 0.00002 gm. per 5 gm.

53 grams of protein, 83 grams of fat, and 260 grams of carbohydrate. The diet for the study of the calcium retention of leaf lettuce consisted of the ordinary strain of Grand Rapids leaf lettuce, lean round beef, soda crackers, butter-fat, and sucrose. In the milk study the diet was the same except that pasteurized whole milk replaced the leaf lettuce, and apples were added. Distilled water was used *ad libitum* for drinking.

Discussion of the Experimental Lettuce

The leaf lettuce used in the study was the ordinary strain of Grand Rapids lettuce. There were two lots, one being planted a little earlier than the other. Each lot was grown under the same conditions at the Purdue University greenhouse. "The crop of lettuce used in the experiment was a part of the second crop which had been grown on the soil after it was sterilized. Before sterilization, a very heavy application of stable manure was spaded into the soil. There was no commercial fertilizer added to the soil."[1]

Enough mature lettuce for a 3-day period was gathered at one time. Each leaf was freed from adhering soil and moisture by use of a towel. Each day's supply for each subject was weighed, placed in a dampened cotton bag which was in turn placed in a heavy brown paper bag, and kept in a refrigerator to prevent wilting. At the same time a thousand gram sample was weighed and allowed to dry in the air in preparation for the calcium analysis.

Approximately one-third of a day's supply was eaten at each meal. It was washed in distilled water before consumption. The daily weighed amount of lettuce was measured by volume and was found to be about a peck. At meal time the lettuce was placed in a bowl on the table and one or two leaves were rolled together and eaten like celery. There was no difficulty in consuming this amount. The water intake, however, was decreased during the lettuce experimental period. It was noted by the subjects that the lettuce was of excellent quality, tasted sweet, and that it contained little cellulose as compared with head lettuce.

The calcium content of the lettuce is given in Table II. From the time that the first lettuce was gathered until the end of the experiment, the weather was cool enough to prevent the spoiling of the mature lettuce left in the soil for a long period.

The calcium from the leaf lettuce furnished 93.0 per cent of the total

[1] Information furnished by Professor E. C. Stair, Department of Horticulture, Purdue University.

TABLE II
CALCIUM CONTENT OF LEAF LETTUCE

Date	Calcium
1932	per cent (Wet basis)
March 9	0.071
March 13	0.075
March 23	0.085*
March 25	0.073
March 28	0.082
March 31	0.079

* 6 analyses, all others in triplicate.

calcium of the diet for one subject and 92.9 per cent of the total calcium for the other. The calcium from the milk furnished 88.4 per cent of the total calcium of the diet for one subject and 88.3 per cent for the other.

The milk, consumed as purchased, was obtained fresh each day from the Purdue University creamery.

A sufficient amount of food, with the exception of lettuce, milk, butter, and meat, was purchased at one time for both experiments. The meat was lean round of beef purchased from the local market in two lots. It was trimmed of fat, ground, and well mixed. Samples were taken for calcium analysis. A day's supply for each subject was weighed for the entire experimental period, wrapped in oil paper, and kept in cold storage. The meat was baked in casseroles in a hot oven from 10 to 20 minutes, according to the preference of the subject. It was eaten from the same container in which it was baked in order to avoid loss. The apples, of the winesap variety, chosen for flavor, were purchased from the local market and were kept in cold storage. To meet the caloric requirement, sugar was eaten as pure cane sugar made into a flavored paste. The daily supply of sucrose was evenly divided for the three meals. The protein of the diet was of high biological value and was consumed in the amount of at least one gram per kilogram of body weight. The torsion balance used for the weighing of the food consumed had its weights checked to the second decimal place with quantitative weights.

For the calcium determinations weighed samples of leaf lettuce of a 3-day gathering (previously dried in the air), pasteurized milk (a composite sample for every three days), cored apple, lean ground beef, and soda crackers were dried in an electric oven and then ashed in porcelain crucibles according to the official method (15) using an electric muffle furnace, kept between 400–450°C.

TABLE III
CALCIUM BALANCE (SIX DAYS)

Subject	Experimental period	Calcium Intake		Calcium Output			Balance	Balance per day	Balance per kg. per day
		Total	Per kg. body weight per day	Urine	Feces	Total			
		gm.	mg.	gm.	gm.	gm.	gm.	gm.	mg.
Subject I Weight 52.3 kg.	Lettuce, leaf	3.180	10.1	0.508	1.843	2.351	+0.829	+0.138	+2.6
	Milk, whole pasteurized	3.060	9.8	0.850	2.119	2.969	+0.091	+0.015	+0.3
Subject II Weight 50.2 kg.	Lettuce, leaf	3.114	10.3	0.219	1.860	2.079	+1.035	+0.173	+3.4
	Milk, whole pasteurized	3.030	10.1	0.638	2.201	2.839	+0.191	+0.032	+0.6

Urine and feces of the 3-day preliminary period were not collected. The first collection of the excreta was made the first day of the experimental period. The feces were dried and ashed for calcium analysis.

Calcium of food, feces, and urine was determined by McCrudden's (16) method with the pH value adjusted according to Shohl and Pedley (17). The 0.01N and 0.05 N $KMnO_4$ solutions were prepared and standardized according to Halverson and Bergeim (18). The potassium permanganate solution used in any series of calcium determinations was restandardized before the titrations for calcium were made. For titrations U. S. Bureau of Standard burettes were used. Redistilled water was used for the preparation of all solutions and for rinsing of all apparatus used in making the analyses. The chemicals used were secured from the Mallinckrodt Chemical Works. The modified calcium method was subjected to preliminary testing for the recovery of calcium from known solutions. Determinations of calcium were made in triplicate.

Discussion and Results

The calcium balances as given in Table III show a daily average balance of +0.138 grams for Subject I and a daily average balance of +0.173 grams for Subject II on a diet in which ordinary leaf lettuce of the Grand Rapids strain was used; and the calcium balances show a daily average balance of +0.015 grams for Subject I and a daily average balance of +0.032 grams for Subject II on a diet in which pasteurized whole milk was used.

A summary of the calcium balance experiments performed at Purdue University is given in Table IV. Subject M. J. has served in six of the studies; subject C.D. in four of the studies; and subject R.J. in two studies. Comparing the calcium retention of the ordinary strain of Grand Rapids fresh leaf lettuce with that of the milk and cheese studies done under similar conditions at Purdue University, it is noted that the calcium retention of the leaf lettuce is high; namely, 2.6 and 3.4 milligrams per kilogram of body weight for subjects M.J. and C.D., respectively.

Was the higher calcium retention due to the hypothesis early stated by Hart, Steenbock, Hoppert, and Humphrey (19) in 1922 that the fresh green plant favored calcium assimilation?

Conclusion

The calcium of the fresh green leafy vegetable, lettuce, which furnished 93 per cent of the total calcium, was superior in its utilization to that of

TABLE IV
SUMMARY OF CALCIUM BALANCE EXPERIMENTS ON THREE YOUNG WOMEN OF SIMILAR AGE, WEIGHT AND HEIGHT AT PURDUE UNIVERSITY

Date	Subject	Body weight	Calcium from food	Calcium Balance (Average Daily) per kilogram of body	
				Intake	Balance
		Raw Centrifuged Milk (Diet Containing High Fat)			
		kg.	per cent	mg.	mg.
Feb. 1929*	M. J.	50.3	93.3	9.8	−0.8
		50.3	93.0	9.4	+1.0
Feb. 1929*	R. J.	50.6	93.3	9.8	+0.9
		50.6	93.0	9.4	−1.9
		Raw Centrifuged Milk (Diet Containing Low Fat)			
Mar. 1929*	M. J.	50.3	94.6	9.6	−0.2
		50.3	94.6	9.6	+0.9
Mar. 1929*	R. J.	50.6	94.6	9.6	−3.0
		50.6	94.6	9.6	−1.2
		American Cheddar Cheese			
Feb. 1931†	M. J.	51.3	86.0	10.2	+0.1
	C. D.	50.2	85.7	10.2	−0.8
		Pasteurized Whole Milk			
Feb. 1931†	M. J.	51.3	87.7	9.4	−0.2
	C. D.	50.2	87.4	9.4	−0.6
April 1932‡	M. J.	52.3	88.4	9.8	+0.3
	C. D.	50.2	88.3	10.1	+0.6
		Leaf Lettuce			
Mar. 1932‡	M. J.	52.3	93.0	10.1	+2.6
	C. D.	50.2	92.9	10.3	+3.4

* Mallon, Jordan, Johnson (6).
† Mallon, Johnson, Darby (8).
‡ Present Study.

pasteurized whole milk when these foods were fed on an approximately equal calcium intake to two healthy young women.

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